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(71) Applicant: BETH ISRAEL HOSPITAL ASSOCIATION [US/US]; 330 Brookline Avenue, Boston, MA 02115 (US).

(72) Inventors: YEO, Kiang-Teck ; 98 Cobleigh Street, Westwood, MA 02090 (US). DVORAK, Harold, F. ; 27 Mason Road, Newton, MA 02159 (US). YEO, Tet-Kin ; 98 Cobleigh Street, Westwood, MA 02090 (US).

(74) Agent: CLARK, Paul, T.; Fish and Richardson, 225 Franklin Street, Boston, MA 02110 (US).

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(54) Title: ASSAY FOR MALIGNANT EFFUSIONS

(57) Abstract

An assay method for determining whether an effusion sample obtained from a human patient is associated with a malignancy, by measuring vascular permeability factor (VPF) in the sample, a VPF level greater than about 30 units (as defined in the description) indicating a likelihood that the sample is a malignant effusion.

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ASSAY FOR MALIGNANT EFFUSIONS

Background of the Invention

This invention relates to assay methods for determining whether effusion samples from human patients are malignant.

Effusions from human patients, e.g., in pleural cavities, can be the result of a variety of diseases including congestive heart failure, cirrhosis of the liver, and pneumonia. However, such effusions may also be the result of a malignancy. Consequently, the need exists for a method to determine whether a given effusion sample may be malignant.

Vascular permeability factor (VPF) is a highly conserved 34-42 kD protein secreted by a variety of tumor cells that has been isolated from serum-free culture medium of carcinoma and sarcoma tumor cells and from tumor ascites fluids. Antibodies directed against VPF have also been produced. Dvorak et al., U.S. Patent No. 4,456,550, which is incorporated herein by reference, describes both the isolation of VPF and the creation of antibodies against VPF. VPF was first measured in animals using the Miles assay, Miles and Miles, J. Physiol. (Lond), 118:228-57 (1952), which measures the extravasation of intravenously injected Evans Blue dye into guinea pig dermis in response to intradermal injections of VPF. Senger et al., Science, 219:983-85 (1983). This assay is used to detect VPF in cell-free culture medium of tumor cells as well as in tumor ascites fluid. Id., Senger et al., Cancer Res., 46:5629-32 (1986). This assay is not specific, because it also detects inflammatory mediators other than VPF.

Various general immunoassays are known in the art. For example, "dissociation enhanced lanthanide fluoroimmunoassay" or DELFIA is a "sandwich" type assay using a time-resolved fluorometer and a lanthanide chelate as a label. Soini et al., Clin. Chem., 29:65-8 (1983); Hemmila et al., Anal. Biochem., 137:335-43 (1984); Hemmila, Clin. Chem., 31:359-70 (1985); Soini et al., CRC Crit Rev. Anal. Chem., 18:105-54 (1987).

Summary of the Invention

The inventors have discovered that VPF is associated with malignant effusions and have developed sensitive and specific assay methods to precisely measure VPF in effusion samples. The VPF immunofluorometric assay of the invention has a minimal detection limit of 0.35 units (as defined below) and is about thirty times more sensitive than the Miles permeability assay. The immunoassay is more precise and simpler to perform, is readily automatable, and can measure large numbers of specimens rapidly and inexpensively. In addition, this assay has a potentially important diagnostic utility as a test for tumor metastases by assaying the effusions in the pleural and peritoneal cavities of human patients.

In general, the invention features an assay method for determining whether an effusion sample obtained from a human patient is associated with a malignancy, comprising measuring vascular permeability factor (VPF) in the sample, a VPF level greater than about 30 units (as defined below) indicating a likelihood that the sample is a malignant effusion.

The invention also features a simple, sensitive, and specific immunofluorometric assay for VPF in a human effusion sample to determine whether the sample is a malignant effusion. This assay employs the following steps: immobilizing a first antibody against a first portion of VPF on a surface, applying the effusion

sample to the immobilized first antibody, incubating the sample for a time and at a temperature sufficient to allow this first antibody to bind to VPF in the sample, washing the surface for a time sufficient to remove unbound VPF, applying a second labeled antibody which is generated against a second portion of VPF to the surface and VPF bound to the immobilized first antibody, incubating the sample for a time and at a temperature sufficient to allow the second antibody to bind to VPF bound to the first antibody, washing the surface for a time sufficient to remove any unbound second antibody, and measuring the amount of label that is bound to the surface to determine the amount of VPF in the sample, a level greater than about 30 units indicating that the sample is a malignant effusion. The term "surface" includes any microtiter plate well, test tube, plate, or other surface to which the first antibodies of the invention can bind.

In preferred embodiments, a pleural or peritoneal effusion is the sample; labeling of the second anti-VPF antibody is carried out with a Europium chelate; and the second antibody used in the test is to a 26-amino acid sequence of the N-terminus of human VPF; i.e., APMAEGGGQNHH-EVVKFMDVYQRSYC.

In further preferred embodiments, the first antibody is to a 20-amino acid sequence of the C-terminus of guinea pig VPF, i.e., YKARQLELNERTCRCDKPRR.

Other features and advantages of the invention will be apparent from the description of the preferred embodiments, and from the claims.

Detailed Description

The drawings will first briefly be described.

Drawings

Fig. 1 is a chromatographic profile of a Europium labeled antibody to the N-terminus of VPF.

Figs. 2A and 2B are graphs showing the optimization curve of the titer of labeled antibody to the C-terminus of VPF.

Figs. 3A and 3B are graphs showing the optimization curve of the titer of an antibody to the N-terminus of VPF.

Figs. 4A and 4B are graphs showing the sensitivity and intra-assay coefficient of variation (CV) of the immunofluorometric VPF assay of the invention.

Fig. 5. is a graph showing the specificity of the immunofluorometric VPF assay of the invention.

Fig. 6 is a graph showing the correlation of the VPF immunoassay of the invention and the Miles assay.

Fig. 7 is a graph showing the kinetics of VPF production in line 1 tumor cells injected in guinea pigs.

Fig. 8 is a graph showing the kinetics of VPF production in line 10 tumor cells injected in guinea pigs.

Fig. 9 is a graph showing calibration curves for the VPF fluoroimmunoassay for both human and guinea pig assays.

Fig. 10 is a chart showing VPF levels in patients with various pathological conditions of fluid accumulation.

A Method for Carrying Out the Invention

Reagents and Equipment: DELFIA™ Eu^{3+} labeling kits are available from Pharmacia-LKB Nuclear Inc. (Gaithersburg, MD). These kits contain 0.2 mg labeling reagent (N^1-[p-isothiocyanatobenzyl]-diethylenetriamine-N^1, N^2, N^3-tetraacetate-Eu^{3+}), 100 nmol/L Eu^{3+} standard,

highly-purified BSA (75 g/L in Tris-HCL, pH 7.8, 0.5 g/L NaN_3) stabilizer, enhancement solution (15 μmol/L 2-napthoyltrifluoroacetone, 50 μmol/L tri-n-octylphosphine oxide, 100 mmol/l acetic acid, 6.8 mmol/L potassium hydrogen phthalate, 1.0 g/L Triton X-100 detergent), assay buffer (Tris-HCl, pH 7.8 solution containing BSA, bovine gamma globulin, Tween 40, diethylenetriamine-pentaacetic acid, 0.5 g/L NaN_3), and wash concentrate solution (25-fold concentration of Tris-HCl/NaCl, pH 7.8, Tween 20)(Soini et al., Clin. Chem., 29:65-8 (1983); Hemmila et al., Anal. Biochem., 137:335-43 (1984); Hemmila, Clin. Chem., 31:359-70 (1985); Soini et al., CRC Crit. Rev. Anal. Chem., 18:105-54 (1987)). PD-10 columns, Sepharose CL-6B, and Sephadex G-50 are available from Pharmacia LKB Biotechnology (Piscataway, NJ). Macrosolute concentrators are available from Amicon (Danvers, MA). Maxisorp microtiter plates and strips (96-well) are available from Nunc Inc. (Naperville, IL). Serum-free media (HL-1) is available from Ventrex Laboratories Inc. (Portland, ME), and hemoglobin crystals are available from Sigma Chemical Co. (St. Louis, MO). The "GammaGone" IgG removal device described below is from Genex Corporation (Gaithersburg, MD).

Buffers: The labeling buffer is 50 mmol/L $NaHCO_3$, pH 8.5, containing 9 g/L NaCl. The elution buffer is 50 mmol/L Tris-HCl, pH 7.8, containing 9 g/L NaCl and 0.5 g/L NaN_3. The coating buffer is phosphate-buffered saline (PBS), pH 7.0, and the blocking reagent is 30 g/L hemoglobin solution.

Polyclonal Antibodies: Antibodies were raised against two synthetic peptides that correspond to the N- and C-termini of guinea pig VPF (designated N-IgG and C-IgG, respectively). In the single letter code, the 25-amino acid sequence of the guinea pig N-terminus of VPF is APMAEGEQK- PREVVKFMDVYKRSYC, and the 20-amino acid

sequence of the C-terminus of guinea pig VPF is (Y)KARQLELNERTCRCDKPRR (the Y amino acid is attached only for coupling purposes). Keck et al., Science, 246:1309-12 (1989). Both peptides were synthesized by Multiple Peptide Systems (San Diego, CA) using standard synthesis techniques and were used to generate antibodies in rabbits as described by Senger et al., Can. Res., 50:1774-78 (1990), except that the C-terminal peptide was coupled to keyhole limpet hemocyanin (KLH) with bis-diazo benzidine. The antibodies (N-IgG and C-IgG) were affinity-purified from rabbit antisera using the respective peptides coupled to CNBr-Sepharose (Pharmacia LKB, Piscataway, NJ). Bound antibodies were eluted from Sepharose-peptide columns with 0.1 mol/L glycine, pH 2.5, and the activity against each peptide was determined by an ELISA method as described by Engvall et al., Methods Enzymol., 70:419-39 (1980).

Briefly, a solution of 1 g/L peptide in 10 mmol/L NaCl, 10 mmol/L Tris, pH 8.5, was used to coat a 96-well microtiter plate. After blocking with 100 g/L normal human serum in PBS, the respective anti-peptide IgG solution (200-fold, 2,000-fold, 10,000-fold dilution) was added. Antibody binding was detected with a peroxidase-labeled, goat anti-rabbit antibody (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) with 2,2-amino-di-[3-ethyl-benzthiazoline sulfonate] as the enzyme substrate. Color development was determined in a THERMOmax™ microplate reader at 405 nm (Molecular Devices, Menlo Park, CA). All affinity-purified IgG preparations retained strong anti-peptide activities down to 10,000-fold dilution. Moreover, both N-IgG and C-IgG (when bound to protein A-Sepharose) efficiently adsorbed VPF from solution, as determined by the Miles vessel permeability assay.

In addition, the method of obtaining antibodies described in Dvorak et al., U.S. Patent No. 4,456,550, which is incorporated herein by reference, may also be used.

Eu^{3+}-labeling of N-IgG: Europium-labeling of the affinity-purified N-IgG was performed according to the DELFIA™ kit protocol with the following modifications. Affinity-purified antisera were pooled and concentrated to about 0.5 g/L using an Amicon macrosolute concentrator. The PD-10 column was pre-equilibrated with 40 ml of labeling buffer, and 2 ml of the antisera (0.5 g/L) was loaded on the column. The column was rinsed with labeling buffer, 1.0 ml fractions were collected, and the absorbance at 280 nm was measured on a Hitachi U-2000 spectrophotometer (Hitachi Instruments Inc., Danbury, CT 06810). Fractions corresponding to peak absorbance were pooled and concentrated to approximately 1 ml, which typically contained 1.5 g/L IgG concentration (an absorptivity value of 1.34 for 1 g/L of IgG was used to calculate IgG concentration). 1.0 ml of the IgG solution was added to 0.2 mg labeling reagent (containing the Eu^{3+}- chelate), and mixed gently on a rotator for 16 h at room temperature.

Purification of Eu^{3+}-labeled IgG: Sepharose CL-6B was poured into a 1.5 cm x 30 cm column to a height of 18 cm. Next preswollen Sephadex G-50 was added to a height of 28 cm and the column was equilibrated with 180 ml of elution buffer. The Eu^{3+}-IgG reaction mixture was added and fractionated on this column. The column was rinsed with elution buffer and sixty 1 ml fractions were collected and their absorbances measured at 280 nm. A small aliquot of each fraction was diluted 10,000-fold with the enhancement solution and the fluorescence was determined on a 1232 DELFIA time-resolved fluorometer (Pharmacia Diagnostics, Fairfield, NJ) using a pulsed

xenon flash at 340 nm and electronic gating to detect fluorescence at 613 nm between 400-800 μs after the excitation flash.

Characterization of Eu^{3+}-labeled N-IgG: Fractions corresponding to peak IgG absorbance (280 nm) and fluorescence were pooled (usually, fractions 25 to 33), and the resulting absorbance (280 nm) and fluorescence (10,000-fold dilution) determined. The calculation of the yield of Eu^{3+}/IgG was determined as described in the DELFIA™ kit protocol (typically, 10 Eu^{3+}/IgG). To increase the stability of the Eu^{3+}-labeled N-IgG antibody, purified BSA was added to a final concentration of 1.0 g/L.

The Sepharose 6B/Sephadex G-50 chromatographic profile in Fig. 1 shows two distinct peaks; the first peak (I) corresponded to Eu^{3+}-labeled N-IgG, and the second peak (II) represented unreacted Eu^{3+}-chelate. Fig. 1 shows absorbance at 280 nm (○) and fluorescence (●). Typical labeling yield is approximately 10 Eu^{3+} per IgG. For this reason, we showed in a separate experiment that >90% of the fluorescence associated with peak I could be removed by an IgG-removing device (Gammagone device), indicating that peak I was comprised mainly of Eu^{3+}-labeled N-IgG. Fractions 25-33, corresponding to Eu^{3+}-IgG, were pooled and the corrected protein concentration was determined to be 115 mg/L (using an absorptivity value of 1.34 g/L for IgG at 280 nm, with corrections made for absorbance of the thiourea bonds of about 0.008 A/μmol/L). The specific activity of the Eu^{3+}-labeled N-IgG was calculated to be approximately 10 Eu^{3+}/IgG, using a 1 nmol/L Eu^{3+} standard as described in the DELFIA kit protocol.

Coating of microtiter strips: 50 μl of a 50-fold dilution of C-IgG (stock concentration of 0.64 g/L in PBS) was added to each well of the microtiter strips, and

the plate incubated overnight at 4°C on a shaker. This is the so-called "first" antibody. Thereafter, the wells were washed six times with DELFIA™ wash buffer, and blocked by incubation with a 30 g/L hemoglobin solution at 20°C for 2 h with gentle shaking. Plates were washed six times with DELFIA™ wash buffer prior to use.

Line 10-cell cultures: Guinea pig line 10 tumor cells were grown as suspension cultures in serum-free defined medium HL-1 as described previously in Yeo et al., Biochem. Biophys. Res. Comm., 179:1568-75 (1991). Conditioned line 10 medium, which contains large amounts of VPF, was centrifuged and frozen at -70°C to serve as calibrators.

Immunoassay procedure: Freshly coated microtiter strips were used on the same day to assay VPF. 50 μL of various dilutions of line 10 conditioned media (using HL-1 medium as the diluent) was added to each well and incubated at 20°C for 2 h with gentle shaking. After six washes with wash buffer, 50 μl of Eu^{3+}-labeled N-IgG (diluted appropriately in assay buffer) was added, incubated for another 2 h at 20°C, and again washed six times. This labeled N-IgG is the so-called "second" antibody in this assay method. Finally, 200 μL of enhancement solution was dispensed into each well, and after 5 min of gentle shaking, the plate was read in the 1232 DELFIA™ fluorometer.

Optimization of the VPF Immunoassay

To determine the optimal dilution of Eu^{3+}-N-IgG, we studied the effect of varying amounts of N-IgG on the VPF binding curve. Microtiter plate wells were immobilized with a constant amount (225 ng/well) of C-IgG. Since pure VPF was not available, line 10 tumor cell conditioned medium, which is rich in VPF, was used to standardize the assay. The same lot of line 10 conditioned medium was used in all experiments. The

concentration of VPF was expressed in arbitrary units; i.e., 100 units is defined as the amount of VPF in our batch of undiluted line 10 tumor cell conditioned medium. If a VPF standard is used that is purified according to the procedures described in Senger et al., Can. Res., 50:1774-78 (1990), one of the "units" defined herein would correspond to a VPF concentration of approximately 20 picograms/ml. A precise relationship between the units defined herein and the VPF concentration can be determined by one of ordinary skill in the art using standard techniques.

We arbitrarily defined "signal" as the fluorescence obtained with 100 units of line 10 conditioned medium, and "noise" as the nonspecific fluorescence associated with HL-1 medium (0 unit). Thus signal-to-noise ratio is defined as $fluorescence_{100\ units}/fluorescence_{0\ unit}$. The effect of varying N-IgG dilution (from 5-fold to 50-fold) is shown in Fig. 2A, which shows the calibration curves at 50-fold (○), 30-fold (◇), 10-fold (△), and 5-fold (□) dilutions of N-IgG, respectively. We determined that 1/50 N-IgG gave a maximal signal-to-noise ratio of 83 (Fig. 2B).

In a separate experiment we studied the effect of varying C-IgG dilution, keeping Eu^{3+}-N-IgG constant at 115 ng/well. C-IgG was coated at 100-fold (○), 75-fold (◇), 50-fold (△), and 30-fold (□) dilutions, respectively, at constant Eu^{3+}-N-IgG concentration. As shown in Fig. 3 (panels A and B), a maximal signal-to-noise ratio of 89 was obtained with 1/30 C-IgG (1000 ng/well). However due to our limited supply of C-IgG, we decided to use a 50-fold dilution of C-IgG (640 ng/well) to coat the microtiter wells; at this concentration, the signal-to-noise ratio was close to maximal at 80. For all subsequent experiments, microtiter plate wells were

coated with 50-fold dilution of C-IgG and bound VPF was detected with 50-fold dilution of Eu^{3+}-N-IgG.

Sensitivity and Intra-assay Coefficient of Variation (CV) of the VPF Immunoassay

To assess the analytical sensitivity of the VPF assay, line 10 conditioned medium corresponding to 0.25 units, 0.50 units, and 1.00 unit were prepared by diluting it with HL-1 medium, and assayed ten times. HL-1 medium devoid of VPF served as the zero standard. The sensitivity, or minimal detectable dose (defined as +2 SD above the zero standard), was about 0.35 units (Fig. 4A), by extrapolation from the standard curve. The intra-assay CV was less than 20% at 0.50 units (Figure 4B).

Specificity of the VPF immunoassay

Since the format of this assay depends on the C-IgG as the "first" or "capture" antibody, and the Eu^{3+}-N-IgG as the "second" or "detector" antibody, we used peptides corresponding to the N- and C-termini of VPF to demonstrate the specificity of the assay. As shown in Fig. 5, inclusion of C-VPF peptide (final concentration of 80 mg/L), N-VPF peptide (final concentration of 80 mg/L), or both peptides in the assay inhibited the binding of VPF in line 10 medium by approximately 80%. In addition when VPF was selectively removed from line 10 conditioned medium (by unlabeled N-IgG followed by incubation wit Protein A-Sepharose and centrifugation), no significant fluorescent signal remained in the supernatant solution. Furthermore, when guinea pig serum, which contains platelet-derived growth factor and other growth factors, was assayed, no VPF was detected (data not shown).

Correlation of VPF Immunoassay With Miles Permeability Assay

Various concentrations of VPF from line 10 medium were prepared and tested in both the Miles permeability assay and the VPF immunofluorometric assay of the invention. For the Miles assay (□), the amount of local dye development due to VPF permeability-enhancing activity was quantitated by absorbance at 620 nm as described in Yeo et al., Biochem. Biophys. Res. Comm., supra. As shown in a comparison of Figs 4 and 6, the VPF immunofluorometric assay of the invention (●) was more sensitive than the Miles permeability assay; at a dose of 0.35 units of VPF, the immunoassay gives values that were significantly different from zero (Fig. 4A). In contrast, the sensitivity of the Miles permeability assay extended to only about 10 units (Fig. 6). There was an excellent linear correlation (R^2=0.94) between the Miles permeability assay and the VPF immunoassay at VPF levels greater than 10 units (Fig. 6, inset).

VPF Assay of Animal Ascites Fluid

Ascites variants of diethylnitrososamine-induced line 1 and line 10 bile duct carcinomas were passaged weekly in the peritoneal cavities of syngeneic strain 2 Sewall-Wright inbred guinea pigs of either sex at 7 day intervals. For determination of the VPF concentration in ascites fluid, plasma, and urine as a function of time following tumor inoculation, guinea pigs were studied at various intervals after i.p. injection of 3 x 10^7 tumor cells. Ascites tumor-bearing or control animals were anesthetized with a mixture of ketamine (15 mg/kg) and rompun (27 mg/kg) given simultaneously i.m. and 5 ml blood samples were collected by cardiac puncture into 0.5 ml of 3.8% sodium citrate. Animals were then sacrificed with ether/CO_2. The peritoneal cavity was then opened by a small incision and 20 ml of Hank's balanced salt

solution (HBSS) was injected i.p. and the contents of the peritoneal cavity were mixed by kneading. The peritoneal contents were recovered to the fullest extent possible by syringe. The total peritoneal fluid volume was recorded and the tumor cells counted. Whenever possible urine was recovered from the bladder by syringe. Blood, peritoneal fluid, and urine were kept on ice and the following inhibitors were added: iodoacetamide (final concentration of 0.37 mg/ml), N-ethylmaleimide (final concentration of 0.25 mg/ml), PMSF (final concentration of 0.35 mg/ml) and aprotinin (final concentration of 210 KIU/ml). Blood, peritoneal fluid, and urine were centrifuged at 160 x g for 20 min at 4°C. The volumes of the resultant plasma, cell-free ascites fluid, and urine were recorded and the samples aliquoted and stored at -80°C until the time of VPF assay.

The two-site time-resolved immunofluorometric assay of the invention was used to assay the guinea pig VPF as described above.

As shown in Figs. 7 and 8, the results show a parallel increase in fluid volume (△), tumor cell number (●), and VPF (■) in the ascites fluids collected from guinea pigs at various times after injection of tumor line 1 and tumor line 10 cells, respectively, into the animals. The insets shows that very little VPF is detected in the plasma (ppp) and the urine (u) of these same animals.

VPF Assay for Human Effusion Samples

In a manner similar to that described above, polyclonal antibodies against the N-terminus of human VPF were produced and labeled with the Europium chelate, and used as the second antibody in the VPF assay method described above. The amino-acid sequence for the N-terminus of human VPF is APMAEGGGQNHHEVVKFMDVYQRSYC. Europium labeled human or guinea pig N-IgG (h-Eu^{3+}-N-IgG

v. g-Eu^{3+}-N-IgG) were used to detect VPF concentration in both human and guinea pig sources of VPF, as shown in Fig. 9. Panel A shows that using a guinea pig source for VPF (line 10 medium), only the guinea pig N-IgG^{Eu3+} (□) binds 5 fold higher than using the human N-IgG^{Eu3+} (■). Panel B shows that using a human source for VPF (human MNNG-HOS cell medium), the human N-IgG^{Eu3+} (◇) binds about 3 fold higher than guinea pig N-IgG^{Eu3+}(◆).

Overall, the results indicate that the specificity of the human N-IgG^{Eu3+}, requires that the correct type of Europium-labeled second antibody be used for the corresponding fluids, i.e., to assay for human VPF, h-N-IgG must be used, and for guinea pig VPF, g-N-IgG must be used. On the other hand, antibodies to the guinea pig VPF C- terminus bound equally well against VPF from human sources, because the C-termini of guinea pig and human VPF are closely related.

Diagnostic Use for Human Patients

Collection of Human Pleural and Peritoneal Effusions

Effusion samples from patients with pathological conditions of fluid accumulations were prepared as follows. The patient's skin was disinfected and a local anesthetic was injected. The pleural space was entered posteriorly in the mid-clavicular line superior to the fifth or sixth rib with a sterile 22-gauge needle and fluid was aspirated into a syringe. Similar aseptic techniques were used to remove peritoneal fluids via a puncture in the right lower quadrant of the abdomen. The fluids were then heparinized, and 1.0 ml aliquots were obtained and centrifuged at 15,000 x G for 1.0 min. in an Eppendorf microcentrifuge. The clear supernatant solutions were immediately frozen and stored at -70°C prior to the VPF assay.

Analysis of VPF Assay Results

VPF levels in the effusion samples were analyzed using a two-sample robust analysis as described in Hoaglin et al., Understanding Robust and Exploratory Data Analysis (John Wiley & Sons, New York, N.Y., 1983). As shown in Fig. 10, VPF levels in patients with malignant cells in effusion fluids are significantly higher than patients without cancer. Overall, these preliminary results suggest that VPF levels in fluids have an important potential diagnostic use to detect cancer. These studies show a strong correlation of high VPF levels with malignant cells in effusion samples, but not necessarily with clinical suspicions of cancer, which may not be definite. Further clinical studies are currently underway to specifically address the use of VPF measurements of effusion samples to diagnose cancer.

Other embodiments are within the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Beth Israel Hospital Association

(ii) TITLE OF INVENTION: ASSAY FOR MALIGNANT EFFUSIONS

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Fish & Richardson
(B) STREET: 225 Franklin Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: U.S.A.
(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM: IBM P.C. DOS (Version 3.30)
(D) SOFTWARE: WordPerfect (Version 5.0)

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(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Clark, Paul T.
(B) REGISTRATION NUMBER: 30,162
(C) REFERENCE/DOCKET NO.: 01948/025WO1

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 542-5070
(B) TELEFAX: (617) 542-8906
(C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His His Glu Val Val Lys
5 10 15
Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys
20 25

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Tyr Lys Ala Arg Gln Leu Glu Leu Asn Gln Arg Thr Cys Arg Cys Asp Lys
5 10 15

Pro Arg Arg

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ala Pro Met Ala Glu Gly Glu Gln Lys Pro Arg Glu Val Val Lys Phe
5 10 15

Met Asp Val Tyr Lys Arg Ser Tyr Cys
20 25

Claims

1. An assay method for determining whether an effusion sample obtained from a human patient is associated with a malignancy, comprising measuring vascular permeability factor (VPF) in the sample, a VPF level greater than a predetermined amount indicating a likelihood that the sample is a malignant effusion.

2. The method of claim 1, comprising

immobilizing a first antibody against a first portion of VPF on a surface,

applying the effusion sample to said immobilized first antibody,

incubating the sample for a time and at a temperature sufficient to allow said first antibody to bind to VPF in said sample,

washing said surface for a time sufficient to remove unbound VPF,

applying a second labeled antibody against a second portion of VPF to said surface and VPF bound to said immobilized first antibody,

incubating the sample for a time and at a temperature sufficient to allow said second antibody to bind to VPF bound to said first antibody,

washing said surface for a time sufficient to remove unbound second antibody, and

measuring the amount of label that is bound to said surface to determine the amount of VPF in the sample, a level greater than about 30 units indicating that the sample is a malignant effusion.

3. The method of claim 1, wherein the effusion sample is a pleural or peritoneal effusion.

4. The method of claim 2, wherein said label is a Europium chelate.

5. The method of claim 2, wherein said second antibody is to a 26-amino acid sequence of the N-terminus of VPF.

6. The method of claim 5, wherein said amino-acid sequence is APMAEGGGQNHHEVVKFMDVYQRSYC.

7. The method of claim 2, wherein said first antibody is to a 20-amino acid sequence of the C-terminus of VPF.

8. The method of claim 7, wherein said amino-acid sequence is YKARQLELNERTCRCDKPRR.

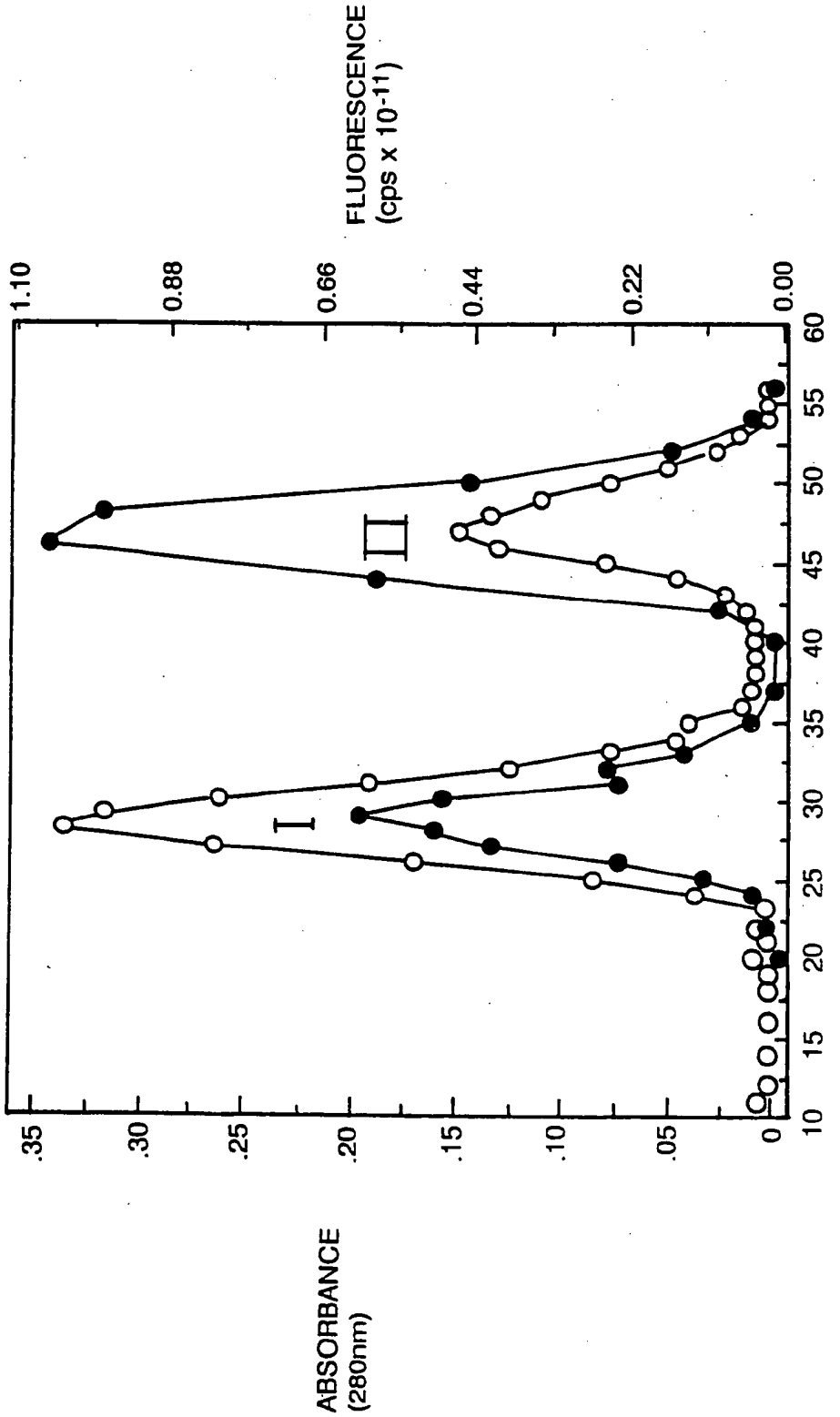
FLUORESCENCE
(cps x 10-11)
1.10
0.88
0.66
0.44
0.22
0.00
I
II
ABSORBANCE
(280nm)
.35
.30
.25
.20
.15
.10
.05
0
10
15
20
25
30
35
40
45
50
55
60
FRACTION

FIG. 1

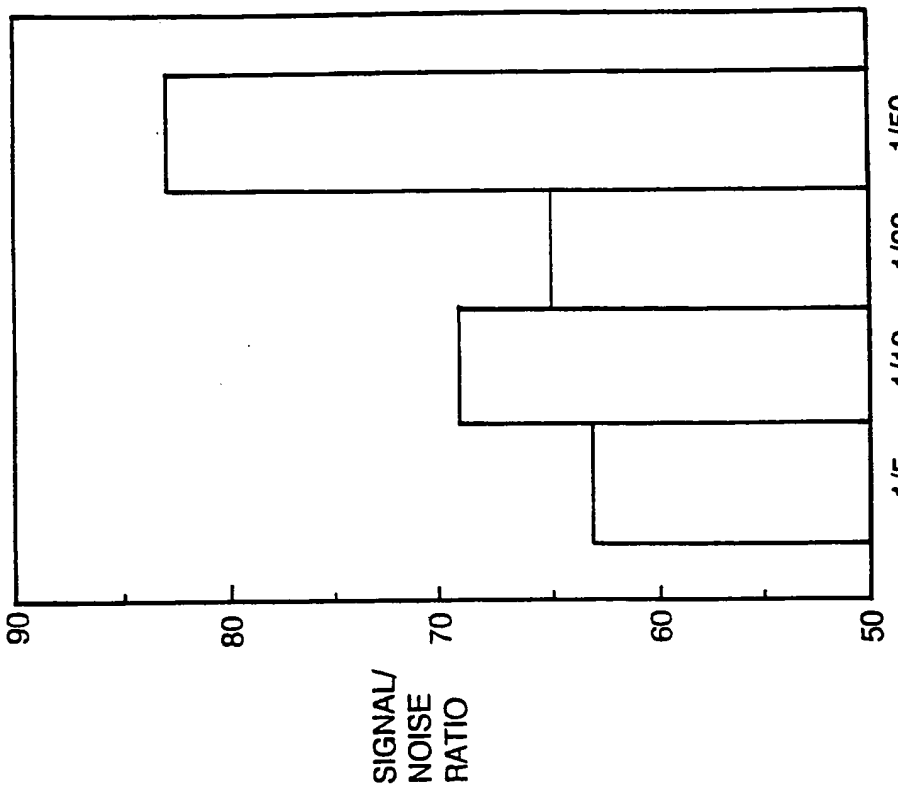
SIGNAL/
NOISE
RATIO
90
80
70
60
50
1/5
1/10
1/30
1/50
ANTIBODY DILUTION

FIG. 2B

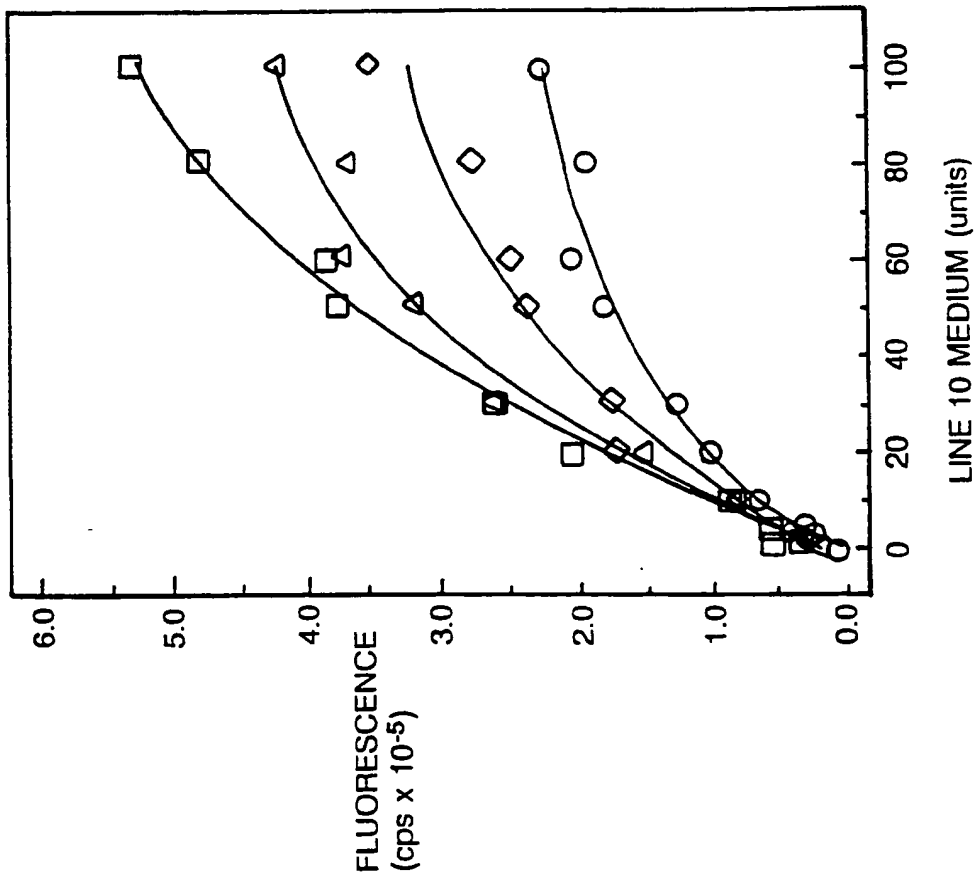
FLUORESCENCE
(cps x 10-5)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
20
40
60
80
100
LINE 10 MEDIUM (units)

FIG. 2A

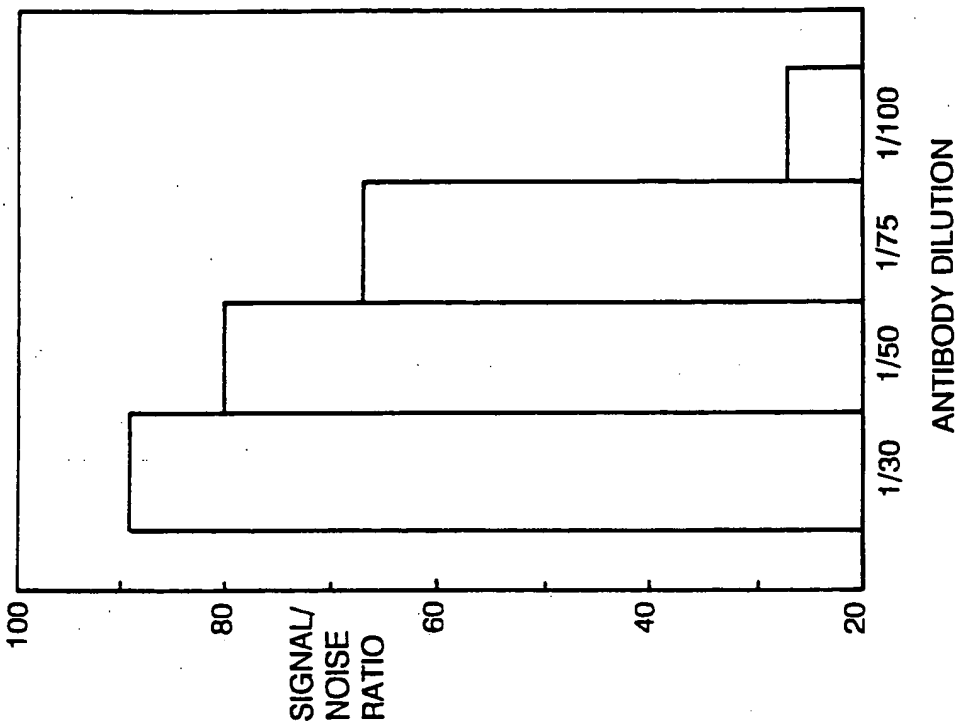

FIG. 3B

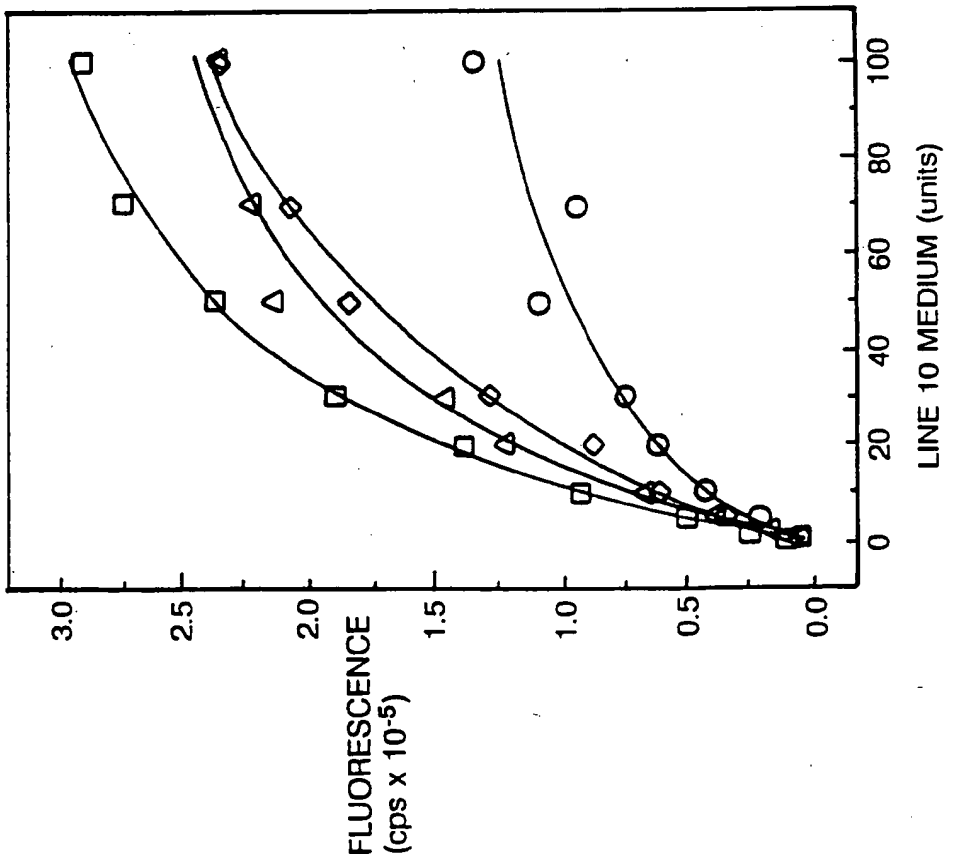

FIG. 3A

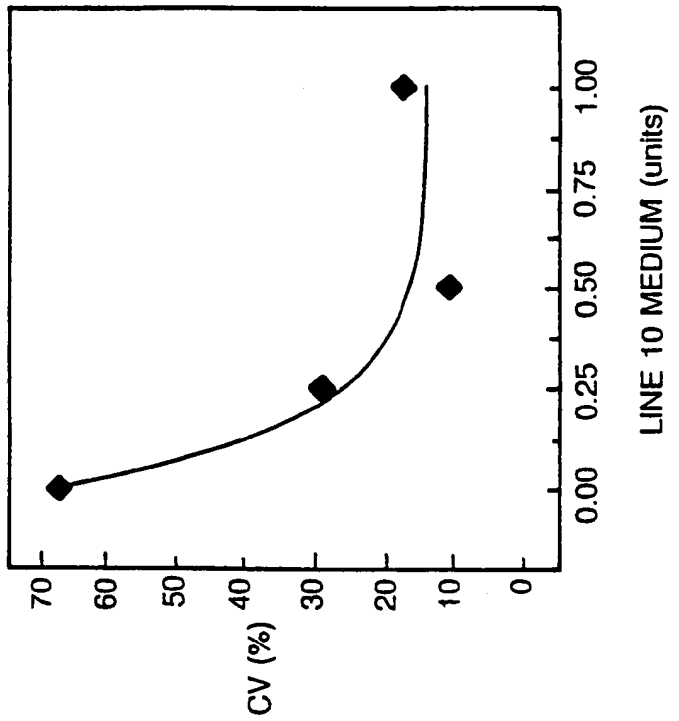
70
60
50
40
30
20
10
0
CV (%)
0.00
0.25
0.50
0.75
1.00
LINE 10 MEDIUM (units)

FIG. 4B

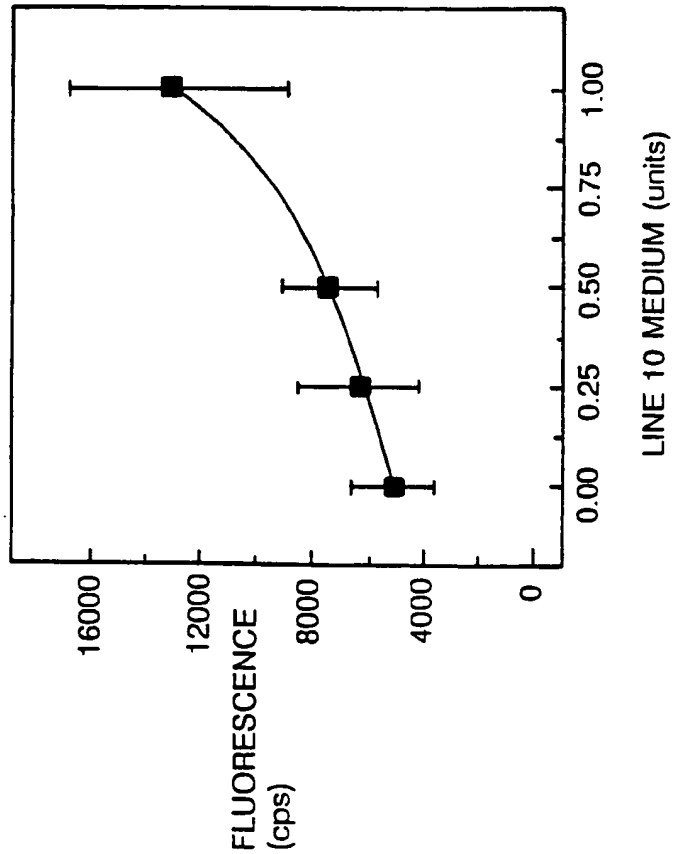
16000
12000
8000
4000
0
FLUORESCENCE (cps)
0.00
0.25
0.50
0.75
1.00
LINE 10 MEDIUM (units)

FIG. 4A

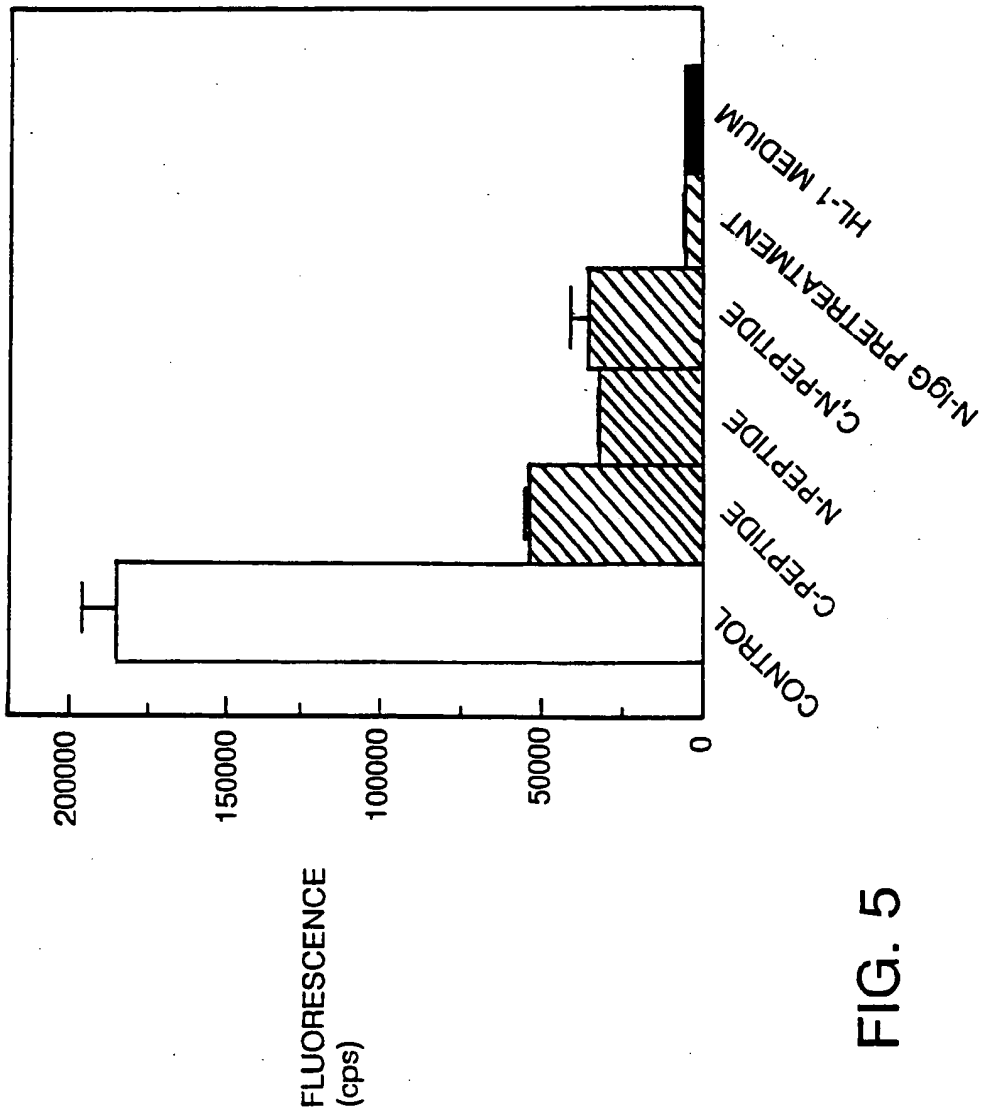
FLUORESCENCE
(cps)
200000
150000
100000
50000
0
CONTROL
C-PEPTIDE
N-PEPTIDE
C,N-PEPTIDE
N-IgG PRETREATMENT
HL-1 MEDIUM

FIG. 5

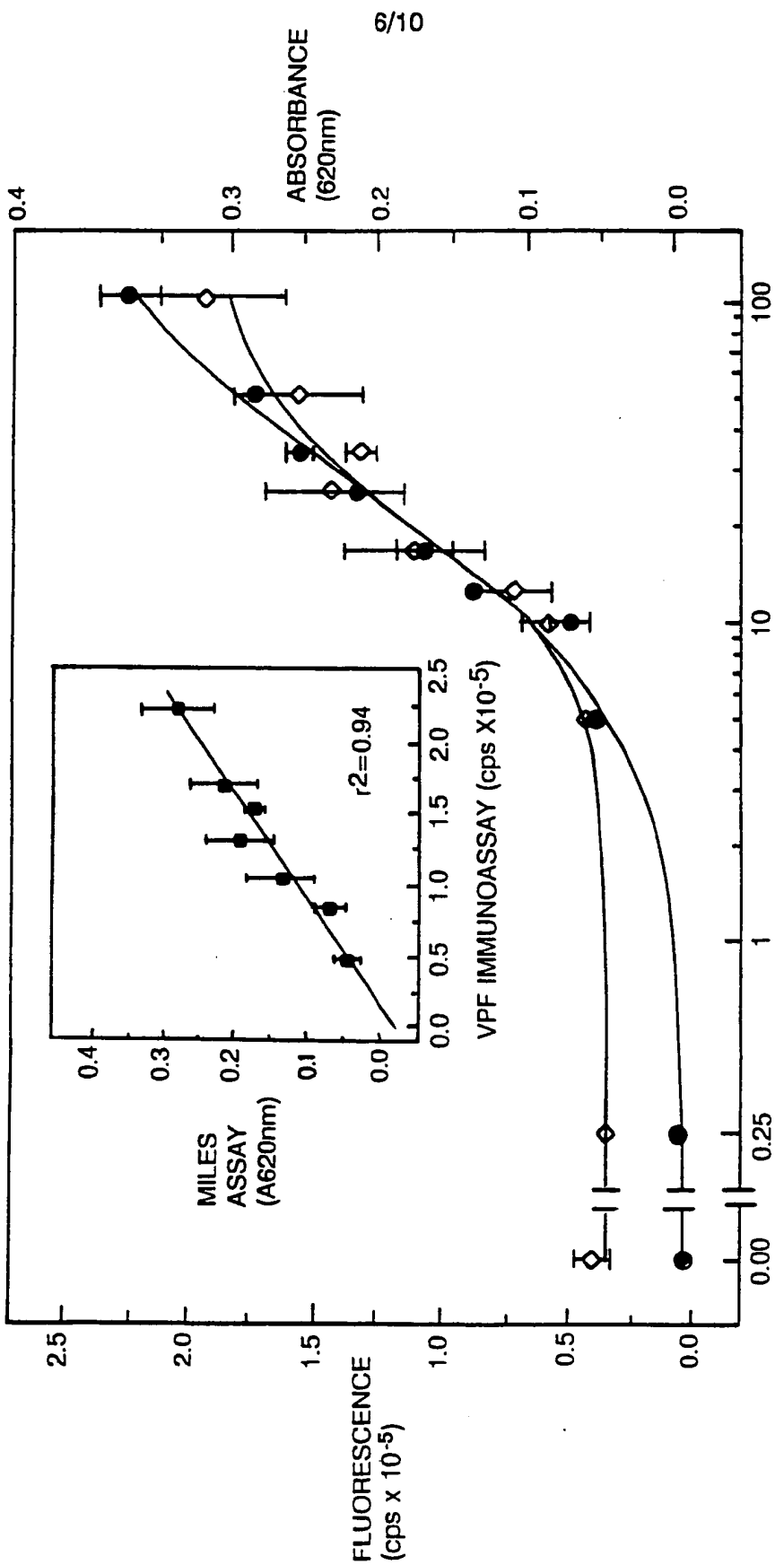
ABSORBANCE (620nm)
0.4
0.3
0.2
0.1
0.0
FLUORESCENCE (cps x 10-5)
2.5
2.0
1.5
1.0
0.5
0.0
0.00
0.25
1
10
100
[VPF] (units)
MILES ASSAY (A620nm)
VPF IMMUNOASSAY (cps X10-5)
r2=0.94

FIG. 6

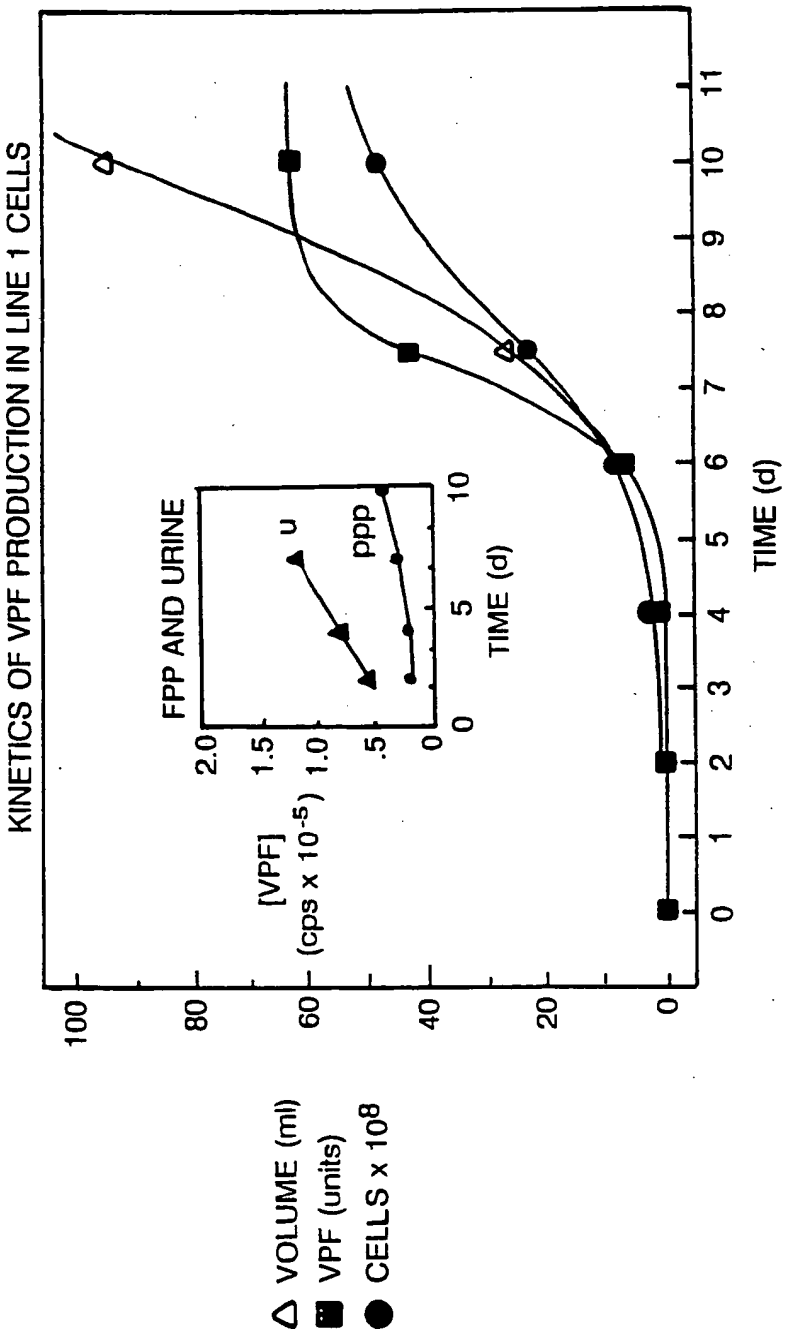

FIG. 7

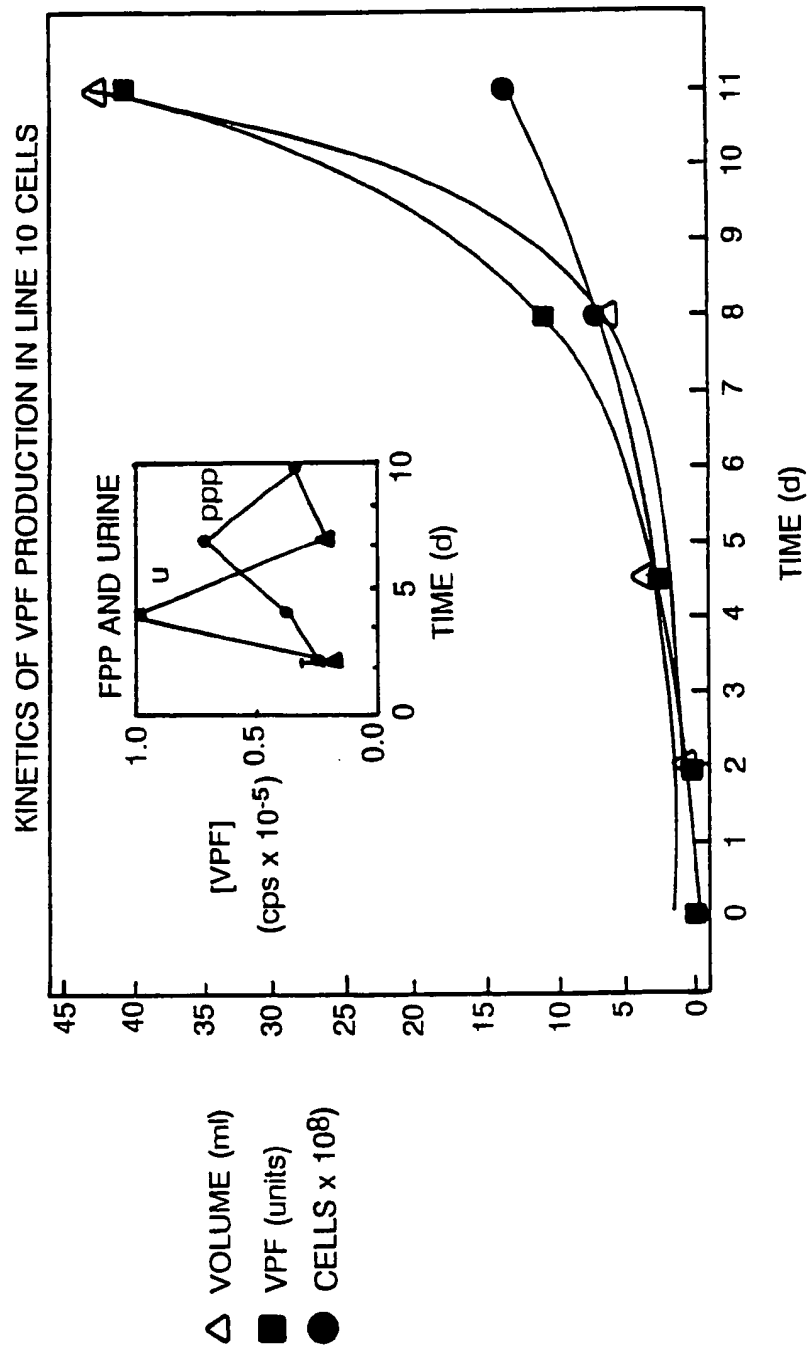
KINETICS OF VPF PRODUCTION IN LINE 10 CELLS
FPP AND URINE
u
ppp
[VPF] (cps x 10-5)
TIME (d)
TIME (d)
VOLUME (ml)
VPF (units)
CELLS x 10^8

FIG. 8

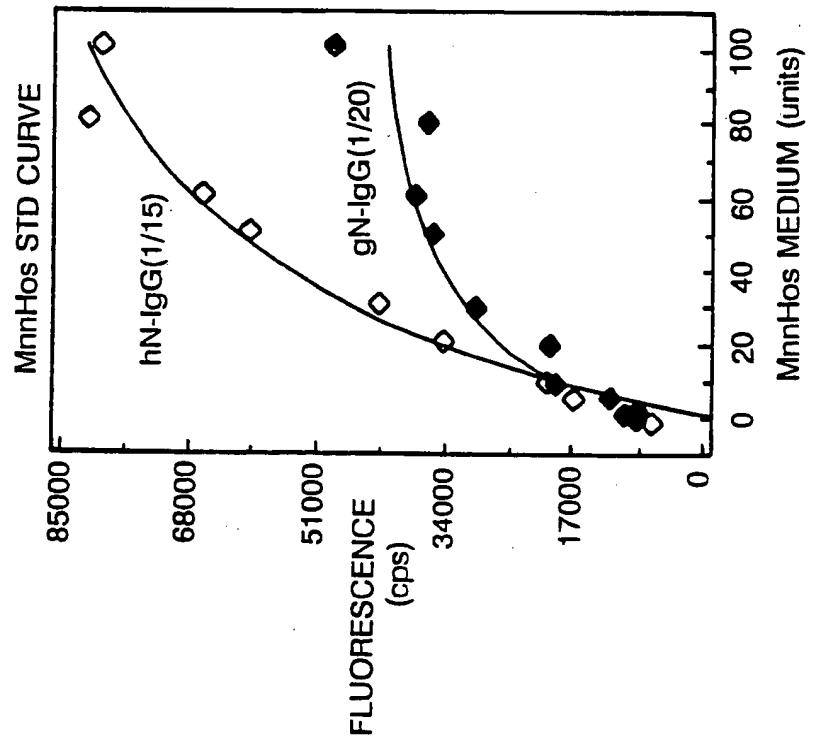

FIG. 9B

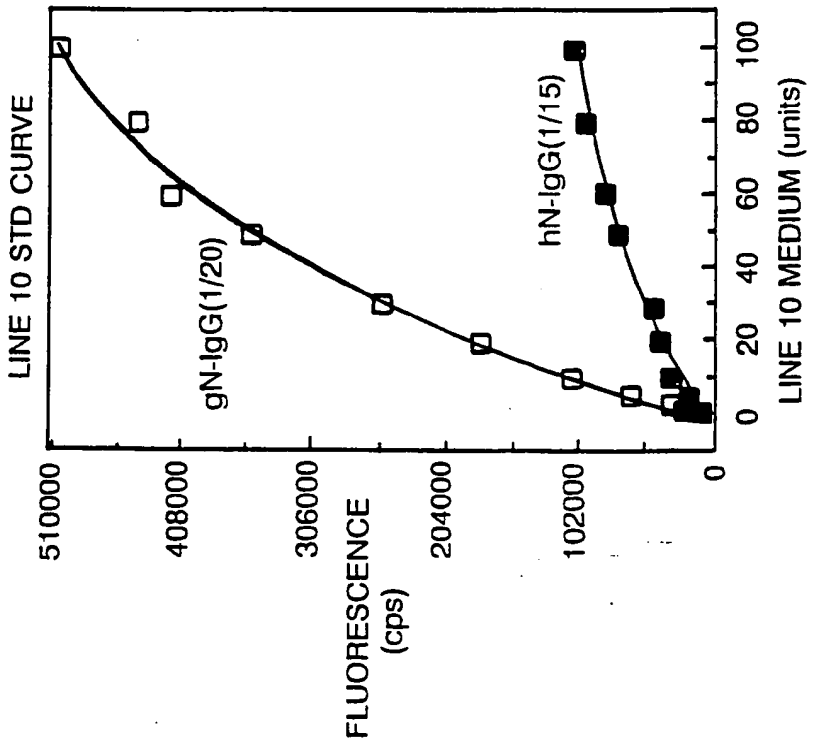

FIG. 9A

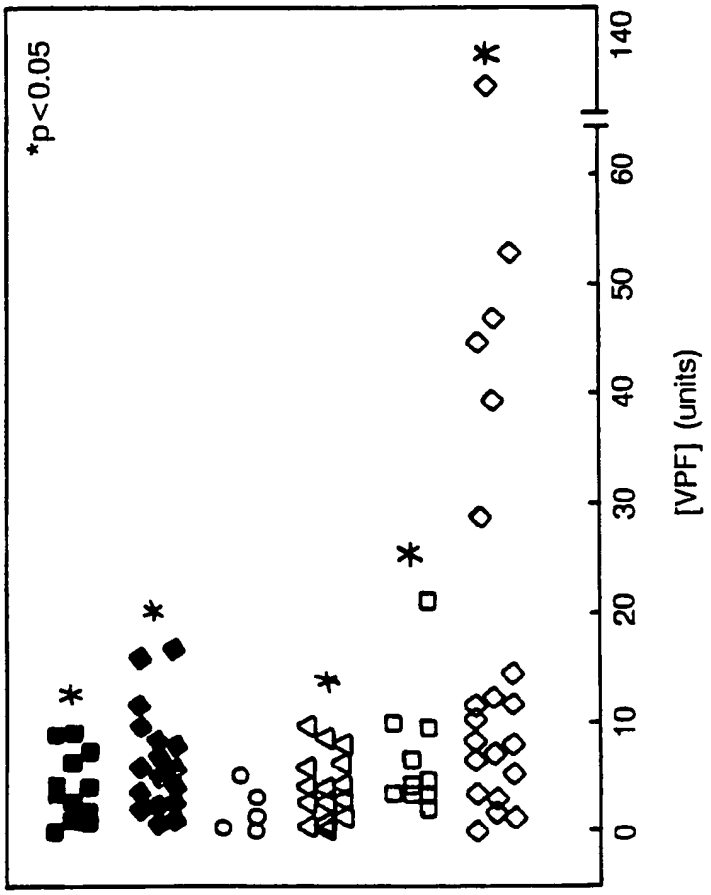

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09068

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/543, 33/50, 33/53
US CL :436/518, 64, 86
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/63; 530/387.1, 388.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, BIOSIS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, Volume 219, issued 25 February 1983 (WASHINGTON, DC, USA), D. R. SENGER ET AL, "Tumor Cells Secrete a Vascular Permeability Factor That Promotes Accumulation of Ascites Fluid", pages 983-985, especially page 985, first column, lines 5-10.	1-8
Y	CANCER RESEARCH, Volume 46, issued November 1986, (USA), D. R. SENGER ET AL., "A Highly Conserved Vascular Permeability Factor Secreted by a Variety of Human and Rodent Tumor Cell Lines", pages 5629-5632, especially page 5629, Abstract and first paragraph of the Introduction, and page 5632, last paragraph.	1-8
Y	US, A, 4,376,110 (DAVID ET AL.) 08 MARCH 1983, especially column 1, line 35 to column 2, line 7.	2, 4-8
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 264, No.33, issued 25 November 1989, (USA), D. T. CONNOLLY ET AL., "Human Vascular Permeability Factor. Isolation from U937 Cells", pages 20017-20024, especially 20019, first column, "Antibodies and Immunoassays" section.	2, 4-8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search 23 November 1992	Date of mailing of the international search report 02 DEC 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer SHERYL K. REILLY Telephone No. (703) 308-3905

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09068

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ACTA CYTOLOGICA, Volume 32, issued September-October 1988, W. W. JOHNSTON, "Fine Needle Aspiration Biopsy Versus Sputum and Bronchial Material in the Diagnosis of Lung Cancer. A Comparative Study of 168 Patients", pages 641-646, especially 645, bridging paragraph between columns 1 and 2.	3
Y	SCANDANAVIAN JOURNAL OF CLINICAL LABORATORY INVESTIGATION, Volume 48, issued 1988, I. HEMMILA, "Lanthanides as probes for time-resolved fluorometric immunoassays", pages 389-399, especially page 392.	4
Y	SCIENCE, Volume 246, issued 08 December 1989, (WASHINGTON, DC, USA), P. J. KECK ET AL., "Vascular Permeability Factor, an Endothelial Cell Mitogen Related to PDGF", pages 1309-1312, especially page 1310, Figure 1.	5-8

CORRECTED VERSION *.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

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(71) Applicant: BETH ISRAEL HOSPITAL ASSOCIATION [US/US]; 330 Brookline Avenue, Boston, MA 02115 (US).

(72) Inventors: YEO, Kiang-Teck ; 98 Cobleigh Street, Westwood, MA 02090 (US). DVORAK, Harold, F. ; 27 Mason Road, Newton, MA 02159 (US). YEO, Tet-Kin ; 98 Cobleigh Street, Westwood, MA 02090 (US).

(74) Agent: CLARK, Paul, T.; Fish and Richardson, 225 Franklin Street, Boston, MA 02110 (US).

(81) Designated States: AU, CA, FI, JP, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: ASSAY FOR MALIGNANT EFFUSIONS

(57) Abstract

An assay method for determining whether an effusion sample obtained from a human patient is associated with a malignancy, by measuring vascular permeability factor (VPF) in the sample, a VPF level greater than about 30 units (as defined in the description) indicating a likelihood that the sample is a malignant effusion.

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ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

ASSAY FOR MALIGNANT EFFUSIONS

Background of the Invention

This invention relates to assay methods for determining whether effusion samples from human patients are malignant.

Effusions from human patients, e.g., in pleural cavities, can be the result of a variety of diseases including congestive heart failure, cirrhosis of the liver, and pneumonia. However, such effusions may also be the result of a malignancy. Consequently, the need exists for a method to determine whether a given effusion sample may be malignant.

Vascular permeability factor (VPF) is a highly conserved 34-42 kD protein secreted by a variety of tumor cells that has been isolated from serum-free culture medium of carcinoma and sarcoma tumor cells and from tumor ascites fluids. Antibodies directed against VPF have also been produced. Dvorak et al., U.S. Patent No. 4,456,550, which is incorporated herein by reference, describes both the isolation of VPF and the creation of antibodies against VPF. VPF was first measured in animals using the Miles assay, Miles and Miles, J. Physiol. (Lond), 118:228-57 (1952), which measures the extravasation of intravenously injected Evans Blue dye into guinea pig dermis in response to intradermal injections of VPF. Senger et al., Science, 219:983-85 (1983). This assay is used to detect VPF in cell-free culture medium of tumor cells as well as in tumor ascites fluid. Id., Senger et al., Cancer Res., 46:5629-32 (1986). This assay is not specific, because it also detects inflammatory mediators other than VPF.

Various general immunoassays are known in the art. For example, "dissociation enhanced lanthanide fluoroimmunoassay" or DELFIA is a "sandwich" type assay using a time-resolved fluorometer and a lanthanide chelate as a label. Soini et al., Clin. Chem., 29:65-8 (1983); Hemmila et al., Anal. Biochem., 137:335-43 (1984); Hemmila, Clin. Chem., 31:359-70 (1985); Soini et al., CRC Crit Rev. Anal. Chem., 18:105-54 (1987).

Summary of the Invention

The inventors have discovered that VPF is associated with malignant effusions and have developed sensitive and specific assay methods to precisely measure VPF in effusion samples. The VPF immunofluorometric assay of the invention has a minimal detection limit of 0.35 units (as defined below) and is about thirty times more sensitive than the Miles permeability assay. The immunoassay is more precise and simpler to perform, is readily automatable, and can measure large numbers of specimens rapidly and inexpensively. In addition, this assay has a potentially important diagnostic utility as a test for tumor metastases by assaying the effusions in the pleural and peritoneal cavities of human patients.

In general, the invention features an assay method for determining whether an effusion sample obtained from a human patient is associated with a malignancy, comprising measuring vascular permeability factor (VPF) in the sample, a VPF level greater than about 30 units (as defined below) indicating a likelihood that the sample is a malignant effusion.

The invention also features a simple, sensitive, and specific immunofluorometric assay for VPF in a human effusion sample to determine whether the sample is a malignant effusion. This assay employs the following steps: immobilizing a first antibody against a first portion of VPF on a surface, applying the effusion

sample to the immobilized first antibody, incubating the sample for a time and at a temperature sufficient to allow this first antibody to bind to VPF in the sample, washing the surface for a time sufficient to remove unbound VPF, applying a second labeled antibody which is generated against a second portion of VPF to the surface and VPF bound to the immobilized first antibody, incubating the sample for a time and at a temperature sufficient to allow the second antibody to bind to VPF bound to the first antibody, washing the surface for a time sufficient to remove any unbound second antibody, and measuring the amount of label that is bound to the surface to determine the amount of VPF in the sample, a level greater than about 30 units indicating that the sample is a malignant effusion. The term "surface" includes any microtiter plate well, test tube, plate, or other surface to which the first antibodies of the invention can bind.

In preferred embodiments, a pleural or peritoneal effusion is the sample; labeling of the second anti-VPF antibody is carried out with a Europium chelate; and the second antibody used in the test is to a 26-amino acid sequence of the N-terminus of human VPF; i.e., APMAEGGGQNHH-EVVKFMDVYQRSYC.

In further preferred embodiments, the first antibody is to a 20-amino acid sequence of the C-terminus of guinea pig VPF, i.e., YKARQLELNERTCRCDKPRR.

Other features and advantages of the invention will be apparent from the description of the preferred embodiments, and from the claims.

Detailed Description

The drawings will first briefly be described.

Drawings

Fig. 1 is a chromatographic profile of a Europium labeled antibody to the N-terminus of VPF.

Figs. 2A and 2B are graphs showing the optimization curve of the titer of labeled antibody to the C-terminus of VPF.

Figs. 3A and 3B are graphs showing the optimization curve of the titer of an antibody to the N-terminus of VPF.

Figs. 4A and 4B are graphs showing the sensitivity and intra-assay coefficient of variation (CV) of the immunofluorometric VPF assay of the invention.

Fig. 5. is a graph showing the specificity of the immunofluorometric VPF assay of the invention.

Fig. 6 is a graph showing the correlation of the VPF immunoassay of the invention and the Miles assay.

Fig. 7 is a graph showing the kinetics of VPF production in line 1 tumor cells injected in guinea pigs.

Fig. 8 is a graph showing the kinetics of VPF production in line 10 tumor cells injected in guinea pigs.

Fig. 9 is a graph showing calibration curves for the VPF fluoroimmunoassay for both human and guinea pig assays.

Fig. 10 is a chart showing VPF levels in patients with various pathological conditions of fluid accumulation.

A Method for Carrying Out the Invention

Reagents and Equipment: DELFIA™ Eu^{3+} labeling kits are available from Pharmacia-LKB Nuclear Inc. (Gaithersburg, MD). These kits contain 0.2 mg labeling reagent (N^1-[p-isothiocyanatobenzyl]-diethylenetriamine-N^1, N^2, N^3-tetraacetate-Eu^{3+}), 100 nmol/L Eu^{3+} standard,

highly-purified BSA (75 g/L in Tris-HCL, pH 7.8, 0.5 g/L NaN_3) stabilizer, enhancement solution (15 μmol/L 2-napthoyltrifluoroacetone, 50 μmol/L tri-n-octylphosphine oxide, 100 mmol/l acetic acid, 6.8 mmol/L potassium hydrogen phthalate, 1.0 g/L Triton X-100 detergent), assay buffer (Tris-HCl, pH 7.8 solution containing BSA, bovine gamma globulin, Tween 40, diethylenetriamine-pentaacetic acid, 0.5 g/L NaN_3), and wash concentrate solution (25-fold concentration of Tris-HCl/NaCl, pH 7.8, Tween 20)(Soini et al., Clin. Chem., 29:65-8 (1983); Hemmila et al., Anal. Biochem., 137:335-43 (1984); Hemmila, Clin. Chem., 31:359-70 (1985); Soini et al., CRC Crit. Rev. Anal. Chem., 18:105-54 (1987)). PD-10 columns, Sepharose CL-6B, and Sephadex G-50 are available from Pharmacia LKB Biotechnology (Piscataway, NJ). Macrosolute concentrators are available from Amicon (Danvers, MA). Maxisorp microtiter plates and strips (96-well) are available from Nunc Inc. (Naperville, IL). Serum-free media (HL-1) is available from Ventrex Laboratories Inc. (Portland, ME), and hemoglobin crystals are available from Sigma Chemical Co. (St. Louis, MO). The "GammaGone" IgG removal device described below is from Genex Corporation (Gaithersburg, MD).

Buffers: The labeling buffer is 50 mmol/L $NaHCO_3$, pH 8.5, containing 9 g/L NaCl. The elution buffer is 50 mmol/L Tris-HCl, pH 7.8, containing 9 g/L NaCl and 0.5 g/L NaN_3. The coating buffer is phosphate-buffered saline (PBS), pH 7.0, and the blocking reagent is 30 g/L hemoglobin solution.

Polyclonal Antibodies: Antibodies were raised against two synthetic peptides that correspond to the N- and C-termini of guinea pig VPF (designated N-IgG and C-IgG, respectively). In the single letter code, the 25-amino acid sequence of the guinea pig N-terminus of VPF is APMAEGEQK- PREVVKFMDVYKRSYC, and the 20-amino acid

sequence of the C-terminus of guinea pig VPF is (Y)KARQLELNERTCRCDKPRR (the Y amino acid is attached only for coupling purposes). Keck et al., Science, 246:1309-12 (1989). Both peptides were synthesized by Multiple Peptide Systems (San Diego, CA) using standard synthesis techniques and were used to generate antibodies in rabbits as described by Senger et al., Can. Res., 50:1774-78 (1990), except that the C-terminal peptide was coupled to keyhole limpet hemocyanin (KLH) with bis-diazo benzidine. The antibodies (N-IgG and C-IgG) were affinity-purified from rabbit antisera using the respective peptides coupled to CNBr-Sepharose (Pharmacia LKB, Piscataway, NJ). Bound antibodies were eluted from Sepharose-peptide columns with 0.1 mol/L glycine, pH 2.5, and the activity against each peptide was determined by an ELISA method as described by Engvall et al., Methods Enzymol., 70:419-39 (1980).

Briefly, a solution of 1 g/L peptide in 10 mmol/L NaCl, 10 mmol/L Tris, pH 8.5, was used to coat a 96-well microtiter plate. After blocking with 100 g/L normal human serum in PBS, the respective anti-peptide IgG solution (200-fold, 2,000-fold, 10,000-fold dilution) was added. Antibody binding was detected with a peroxidase-labeled, goat anti-rabbit antibody (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) with 2,2-amino-di-[3-ethyl-benzthiazoline sulfonate] as the enzyme substrate. Color development was determined in a THERMOmax™ microplate reader at 405 nm (Molecular Devices, Menlo Park, CA). All affinity-purified IgG preparations retained strong anti-peptide activities down to 10,000-fold dilution. Moreover, both N-IgG and C-IgG (when bound to protein A-Sepharose) efficiently adsorbed VPF from solution, as determined by the Miles vessel permeability assay.

In addition, the method of obtaining antibodies described in Dvorak et al., U.S. Patent No. 4,456,550, which is incorporated herein by reference, may also be used.

Eu^{3+}-labeling of N-IgG: Europium-labeling of the affinity-purified N-IgG was performed according to the DELFIA™ kit protocol with the following modifications. Affinity-purified antisera were pooled and concentrated to about 0.5 g/L using an Amicon macrosolute concentrator. The PD-10 column was pre-equilibrated with 40 ml of labeling buffer, and 2 ml of the antisera (0.5 g/L) was loaded on the column. The column was rinsed with labeling buffer, 1.0 ml fractions were collected, and the absorbance at 280 nm was measured on a Hitachi U-2000 spectrophotometer (Hitachi Instruments Inc., Danbury, CT 06810). Fractions corresponding to peak absorbance were pooled and concentrated to approximately 1 ml, which typically contained 1.5 g/L IgG concentration (an absorptivity value of 1.34 for 1 g/L of IgG was used to calculate IgG concentration). 1.0 ml of the IgG solution was added to 0.2 mg labeling reagent (containing the Eu^{3+}- chelate), and mixed gently on a rotator for 16 h at room temperature.

Purification of Eu^{3+}-labeled IgG: Sepharose CL-6B was poured into a 1.5 cm x 30 cm column to a height of 18 cm. Next preswollen Sephadex G-50 was added to a height of 28 cm and the column was equilibrated with 180 ml of elution buffer. The Eu^{3+}-IgG reaction mixture was added and fractionated on this column. The column was rinsed with elution buffer and sixty 1 ml fractions were collected and their absorbances measured at 280 nm. A small aliquot of each fraction was diluted 10,000-fold with the enhancement solution and the fluorescence was determined on a 1232 DELFIA time-resolved fluorometer (Pharmacia Diagnostics, Fairfield, NJ) using a pulsed

xenon flash at 340 nm and electronic gating to detect fluorescence at 613 nm between 400-800 μs after the excitation flash.

Characterization of Eu^{3+}-labeled N-IgG: Fractions corresponding to peak IgG absorbance (280 nm) and fluorescence were pooled (usually, fractions 25 to 33), and the resulting absorbance (280 nm) and fluorescence (10,000-fold dilution) determined. The calculation of the yield of Eu^{3+}/IgG was determined as described in the DELFIA™ kit protocol (typically, 10 Eu^{3+}/IgG). To increase the stability of the Eu^{3+}-labeled N-IgG antibody, purified BSA was added to a final concentration of 1.0 g/L.

The Sepharose 6B/Sephadex G-50 chromatographic profile in Fig. 1 shows two distinct peaks; the first peak (I) corresponded to Eu^{3+}-labeled N-IgG, and the second peak (II) represented unreacted Eu^{3+}-chelate. Fig. 1 shows absorbance at 280 nm (○) and fluorescence (●). Typical labeling yield is approximately 10 Eu^{3+} per IgG. For this reason, we showed in a separate experiment that >90% of the fluorescence associated with peak I could be removed by an IgG-removing device (Gammagone device), indicating that peak I was comprised mainly of Eu^{3+}-labeled N-IgG. Fractions 25-33, corresponding to Eu^{3+}-IgG, were pooled and the corrected protein concentration was determined to be 115 mg/L (using an absorptivity value of 1.34 g/L for IgG at 280 nm, with corrections made for absorbance of the thiourea bonds of about 0.008 A/μmol/L). The specific activity of the Eu^{3+}-labeled N-IgG was calculated to be approximately 10 Eu^{3+}/IgG, using a 1 nmol/L Eu^{3+} standard as described in the DELFIA kit protocol.

Coating of microtiter strips: 50 μl of a 50-fold dilution of C-IgG (stock concentration of 0.64 g/L in PBS) was added to each well of the microtiter strips, and

the plate incubated overnight at 4°C on a shaker. This is the so-called "first" antibody. Thereafter, the wells were washed six times with DELFIA™ wash buffer, and blocked by incubation with a 30 g/L hemoglobin solution at 20°C for 2 h with gentle shaking. Plates were washed six times with DELFIA™ wash buffer prior to use.

Line 10-cell cultures: Guinea pig line 10 tumor cells were grown as suspension cultures in serum-free defined medium HL-1 as described previously in Yeo et al., Biochem. Biophys. Res. Comm., 179:1568-75 (1991). Conditioned line 10 medium, which contains large amounts of VPF, was centrifuged and frozen at -70°C to serve as calibrators.

Immunoassay procedure: Freshly coated microtiter strips were used on the same day to assay VPF. 50 μL of various dilutions of line 10 conditioned media (using HL-1 medium as the diluent) was added to each well and incubated at 20°C for 2 h with gentle shaking. After six washes with wash buffer, 50 μl of Eu^{3+}-labeled N-IgG (diluted appropriately in assay buffer) was added, incubated for another 2 h at 20°C, and again washed six times. This labeled N-IgG is the so-called "second" antibody in this assay method. Finally, 200 μL of enhancement solution was dispensed into each well, and after 5 min of gentle shaking, the plate was read in the 1232 DELFIA™ fluorometer.

Optimization of the VPF Immunoassay

To determine the optimal dilution of Eu^{3+}-N-IgG, we studied the effect of varying amounts of N-IgG on the VPF binding curve. Microtiter plate wells were immobilized with a constant amount (225 ng/well) of C-IgG. Since pure VPF was not available, line 10 tumor cell conditioned medium, which is rich in VPF, was used to standardize the assay. The same lot of line 10 conditioned medium was used in all experiments. The

concentration of VPF was expressed in arbitrary units; i.e., 100 units is defined as the amount of VPF in our batch of undiluted line 10 tumor cell conditioned medium. If a VPF standard is used that is purified according to the procedures described in Senger et al., Can. Res., 50:1774-78 (1990), one of the "units" defined herein would correspond to a VPF concentration of approximately 20 picograms/ml. A precise relationship between the units defined herein and the VPF concentration can be determined by one of ordinary skill in the art using standard techniques.

We arbitrarily defined "signal" as the fluorescence obtained with 100 units of line 10 conditioned medium, and "noise" as the nonspecific fluorescence associated with HL-1 medium (0 unit). Thus signal-to-noise ratio is defined as $fluorescence_{100\ units}/fluorescence_{0\ unit}$. The effect of varying N-IgG dilution (from 5-fold to 50-fold) is shown in Fig. 2A, which shows the calibration curves at 50-fold (○), 30-fold (◇), 10-fold (△), and 5-fold (□) dilutions of N-IgG, respectively. We determined that 1/50 N-IgG gave a maximal signal-to-noise ratio of 83 (Fig. 2B).

In a separate experiment we studied the effect of varying C-IgG dilution, keeping Eu^{3+}-N-IgG constant at 115 ng/well. C-IgG was coated at 100-fold (○), 75-fold (◇), 50-fold (△), and 30-fold (□) dilutions, respectively, at constant Eu^{3+}-N-IgG concentration. As shown in Fig. 3 (panels A and B), a maximal signal-to-noise ratio of 89 was obtained with 1/30 C-IgG (1000 ng/well). However due to our limited supply of C-IgG, we decided to use a 50-fold dilution of C-IgG (640 ng/well) to coat the microtiter wells; at this concentration, the signal-to-noise ratio was close to maximal at 80. For all subsequent experiments, microtiter plate wells were

coated with 50-fold dilution of C-IgG and bound VPF was detected with 50-fold dilution of Eu^{3+}-N-IgG.

Sensitivity and Intra-assay Coefficient of Variation (CV) of the VPF Immunoassay

To assess the analytical sensitivity of the VPF assay, line 10 conditioned medium corresponding to 0.25 units, 0.50 units, and 1.00 unit were prepared by diluting it with HL-1 medium, and assayed ten times. HL-1 medium devoid of VPF served as the zero standard. The sensitivity, or minimal detectable dose (defined as +2 SD above the zero standard), was about 0.35 units (Fig. 4A), by extrapolation from the standard curve. The intra-assay CV was less than 20% at 0.50 units (Figure 4B).

Specificity of the VPF immunoassay

Since the format of this assay depends on the C-IgG as the "first" or "capture" antibody, and the Eu^{3+}-N-IgG as the "second" or "detector" antibody, we used peptides corresponding to the N- and C-termini of VPF to demonstrate the specificity of the assay. As shown in Fig. 5, inclusion of C-VPF peptide (final concentration of 80 mg/L), N-VPF peptide (final concentration of 80 mg/L), or both peptides in the assay inhibited the binding of VPF in line 10 medium by approximately 80%. In addition when VPF was selectively removed from line 10 conditioned medium (by unlabeled N-IgG followed by incubation wit Protein A-Sepharose and centrifugation), no significant fluorescent signal remained in the supernatant solution. Furthermore, when guinea pig serum, which contains platelet-derived growth factor and other growth factors, was assayed, no VPF was detected (data not shown).

Correlation of VPF Immunoassay With Miles Permeability Assay

Various concentrations of VPF from line 10 medium were prepared and tested in both the Miles permeability assay and the VPF immunofluorometric assay of the invention. For the Miles assay (□), the amount of local dye development due to VPF permeability-enhancing activity was quantitated by absorbance at 620 nm as described in Yeo et al., Biochem. Biophys. Res. Comm., supra. As shown in a comparison of Figs 4 and 6, the VPF immunofluorometric assay of the invention (●) was more sensitive than the Miles permeability assay; at a dose of 0.35 units of VPF, the immunoassay gives values that were significantly different from zero (Fig. 4A). In contrast, the sensitivity of the Miles permeability assay extended to only about 10 units (Fig. 6). There was an excellent linear correlation (R^2=0.94) between the Miles permeability assay and the VPF immunoassay at VPF levels greater than 10 units (Fig. 6, inset).

VPF Assay of Animal Ascites Fluid

Ascites variants of diethylnitrososamine-induced line 1 and line 10 bile duct carcinomas were passaged weekly in the peritoneal cavities of syngeneic strain 2 Sewall-Wright inbred guinea pigs of either sex at 7 day intervals. For determination of the VPF concentration in ascites fluid, plasma, and urine as a function of time following tumor inoculation, guinea pigs were studied at various intervals after i.p. injection of 3 x 10^7 tumor cells. Ascites tumor-bearing or control animals were anesthetized with a mixture of ketamine (15 mg/kg) and rompun (27 mg/kg) given simultaneously i.m. and 5 ml blood samples were collected by cardiac puncture into 0.5 ml of 3.8% sodium citrate. Animals were then sacrificed with ether/CO_2. The peritoneal cavity was then opened by a small incision and 20 ml of Hank's balanced salt

solution (HBSS) was injected i.p. and the contents of the peritoneal cavity were mixed by kneading. The peritoneal contents were recovered to the fullest extent possible by syringe. The total peritoneal fluid volume was recorded and the tumor cells counted. Whenever possible urine was recovered from the bladder by syringe. Blood, peritoneal fluid, and urine were kept on ice and the following inhibitors were added: iodoacetamide (final concentration of 0.37 mg/ml), N-ethylmaleimide (final concentration of 0.25 mg/ml), PMSF (final concentration of 0.35 mg/ml) and aprotinin (final concentration of 210 KIU/ml). Blood, peritoneal fluid, and urine were centrifuged at 160 x g for 20 min at 4°C. The volumes of the resultant plasma, cell-free ascites fluid, and urine were recorded and the samples aliquoted and stored at -80°C until the time of VPF assay.

The two-site time-resolved immunofluorometric assay of the invention was used to assay the guinea pig VPF as described above.

As shown in Figs. 7 and 8, the results show a parallel increase in fluid volume (▵), tumor cell number (●), and VPF (■) in the ascites fluids collected from guinea pigs at various times after injection of tumor line 1 and tumor line 10 cells, respectively, into the animals. The insets shows that very little VPF is detected in the plasma (ppp) and the urine (u) of these same animals.

VPF Assay for Human Effusion Samples

In a manner similar to that described above, polyclonal antibodies against the N-terminus of human VPF were produced and labeled with the Europium chelate, and used as the second antibody in the VPF assay method described above. The amino-acid sequence for the N-terminus of human VPF is APMAEGGGQNHHEVVKFMDVYQRSYC. Europium labeled human or guinea pig N-IgG (h-Eu^{3+}-N-IgG

v. g-Eu^{3+}-N-IgG) were used to detect VPF concentration in both human and guinea pig sources of VPF, as shown in Fig. 9. Panel A shows that using a guinea pig source for VPF (line 10 medium), only the guinea pig N-IgG^{Eu3+} (□) binds 5 fold higher than using the human N-IgG^{Eu3+} (■). Panel B shows that using a human source for VPF (human MNNG-HOS cell medium), the human N-IgG^{Eu3+} (◇) binds about 3 fold higher than guinea pig N-IgG^{Eu3+}(◆).

Overall, the results indicate that the specificity of the human N-IgG^{Eu3+}, requires that the correct type of Europium-labeled second antibody be used for the corresponding fluids, i.e., to assay for human VPF, h-N-IgG must be used, and for guinea pig VPF, g-N-IgG must be used. On the other hand, antibodies to the guinea pig VPF C- terminus bound equally well against VPF from human sources, because the C-termini of guinea pig and human VPF are closely related.

Diagnostic Use for Human Patients

Collection of Human Pleural and Peritoneal Effusions

Effusion samples from patients with pathological conditions of fluid accumulations were prepared as follows. The patient's skin was disinfected and a local anesthetic was injected. The pleural space was entered posteriorly in the mid-clavicular line superior to the fifth or sixth rib with a sterile 22-gauge needle and fluid was aspirated into a syringe. Similar aseptic techniques were used to remove peritoneal fluids via a puncture in the right lower quadrant of the abdomen. The fluids were then heparinized, and 1.0 ml aliquots were obtained and centrifuged at 15,000 x G for 1.0 min. in an Eppendorf microcentrifuge. The clear supernatant solutions were immediately frozen and stored at -70°C prior to the VPF assay.

Analysis of VPF Assay Results

VPF levels in the effusion samples were analyzed using a two-sample robust analysis as described in Hoaglin et al., Understanding Robust and Exploratory Data Analysis (John Wiley & Sons, New York, N.Y., 1983). As shown in Fig. 10, VPF levels in patients with malignant cells in effusion fluids are significantly higher than patients without cancer. Overall, these preliminary results suggest that VPF levels in fluids have an important potential diagnostic use to detect cancer. These studies show a strong correlation of high VPF levels with malignant cells in effusion samples, but not necessarily with clinical suspicions of cancer, which may not be definite. Further clinical studies are currently underway to specifically address the use of VPF measurements of effusion samples to diagnose cancer.

Other embodiments are within the following claims.

SEQUENCE LISTING

(1) **GENERAL INFORMATION:**

(i) **APPLICANT:** Beth Israel Hospital Association

(ii) **TITLE OF INVENTION:** ASSAY FOR MALIGNANT EFFUSIONS

(iii) **NUMBER OF SEQUENCES:** 3

(iv) **CORRESPONDENCE ADDRESS:**

(A) **ADDRESSEE:** Fish & Richardson
(B) **STREET:** 225 Franklin Street
(C) **CITY:** Boston
(D) **STATE:** Massachusetts
(E) **COUNTRY:** U.S.A.
(F) **ZIP:** 02110-2804

(v) **COMPUTER READABLE FORM:**

(A) **MEDIUM TYPE:** 3.5" Diskette, 1.44 Mb
(B) **COMPUTER:** IBM PS/2 Model 50Z or 55SX
(C) **OPERATING SYSTEM:** IBM P.C. DOS (Version 3.30)
(D) **SOFTWARE:** WordPerfect (Version 5.0)

(vi) **CURRENT APPLICATION DATA:**

(A) **APPLICATION NUMBER:**
(B) **FILING DATE:**
(C) **CLASSIFICATION:**

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 782,350
(B) FILING DATE: 24 October 1991

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Clark, Paul T.
(B) REGISTRATION NUMBER: 30,162
(C) REFERENCE/DOCKET NO.: 01948/025WO1

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 542-5070
(B) TELEFAX: (617) 542-8906
(C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His His Glu Val Val Lys
5 10 15
Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys
20 25

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Tyr Lys Ala Arg Gln Leu Glu Leu Asn Gln Arg Thr Cys Arg Cys Asp Lys
5 10 15
Pro Arg Arg

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ala Pro Met Ala Glu Gly Glu Gln Lys Pro Arg Glu Val Val Lys Phe
5 10 15
Met Asp Val Tyr Lys Arg Ser Tyr Cys
20 25

Claims

1. An assay method for determining whether an effusion sample obtained from a human patient is associated with a malignancy, comprising measuring vascular permeability factor (VPF) in the sample, a VPF level greater than a predetermined amount indicating a likelihood that the sample is a malignant effusion.

2. The method of claim 1, comprising

immobilizing a first antibody against a first portion of VPF on a surface,

applying the effusion sample to said immobilized first antibody,

incubating the sample for a time and at a temperature sufficient to allow said first antibody to bind to VPF in said sample,

washing said surface for a time sufficient to remove unbound VPF,

applying a second labeled antibody against a second portion of VPF to said surface and VPF bound to said immobilized first antibody,

incubating the sample for a time and at a temperature sufficient to allow said second antibody to bind to VPF bound to said first antibody,

washing said surface for a time sufficient to remove unbound second antibody, and

measuring the amount of label that is bound to said surface to determine the amount of VPF in the sample, a level greater than about 30 units indicating that the sample is a malignant effusion.

3. The method of claim 1, wherein the effusion sample is a pleural or peritoneal effusion.

4. The method of claim 2, wherein said label is a Europium chelate.

5. The method of claim 2, wherein said second antibody is to a 26-amino acid sequence of the N-terminus of VPF.

6. The method of claim 5, wherein said amino-acid sequence is APMAEGGGQNHHEVVKFMDVYQRSYC.

7. The method of claim 2, wherein said first antibody is to a 20-amino acid sequence of the C-terminus of VPF.

8. The method of claim 7, wherein said amino-acid sequence is YKARQLELNERTCRCDKPRR.

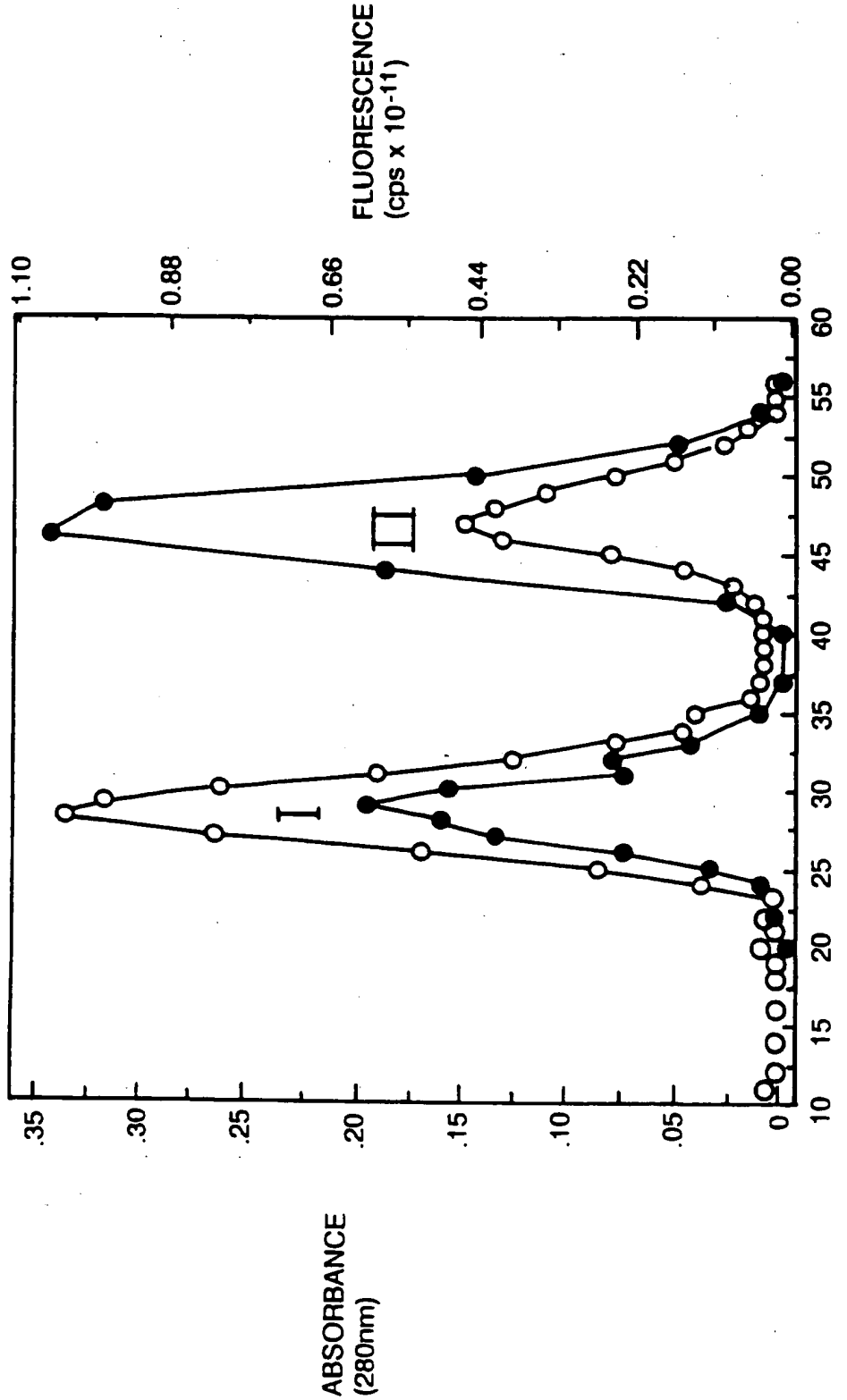

FIG. 1

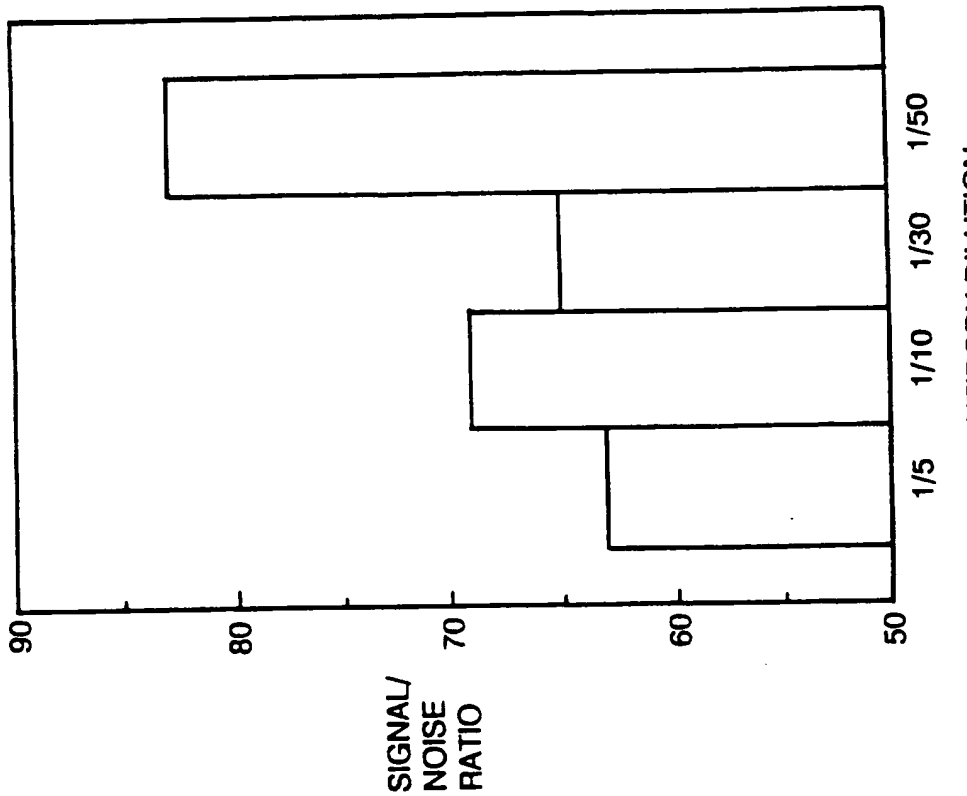
SIGNAL/
NOISE
RATIO
90
80
70
60
50
1/5
1/10
1/30
1/50
ANTIBODY DILUTION

FIG. 2B

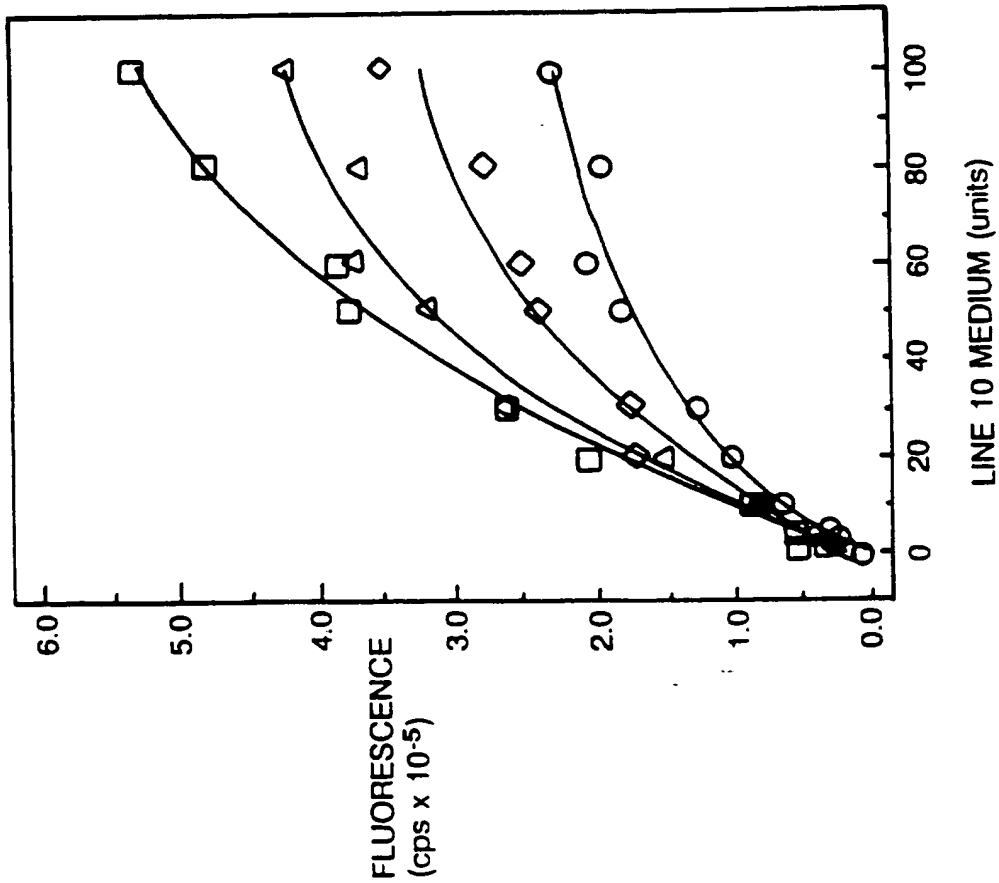
FLUORESCENCE
(cps x 10-5)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
20
40
60
80
100
LINE 10 MEDIUM (units)

FIG. 2A

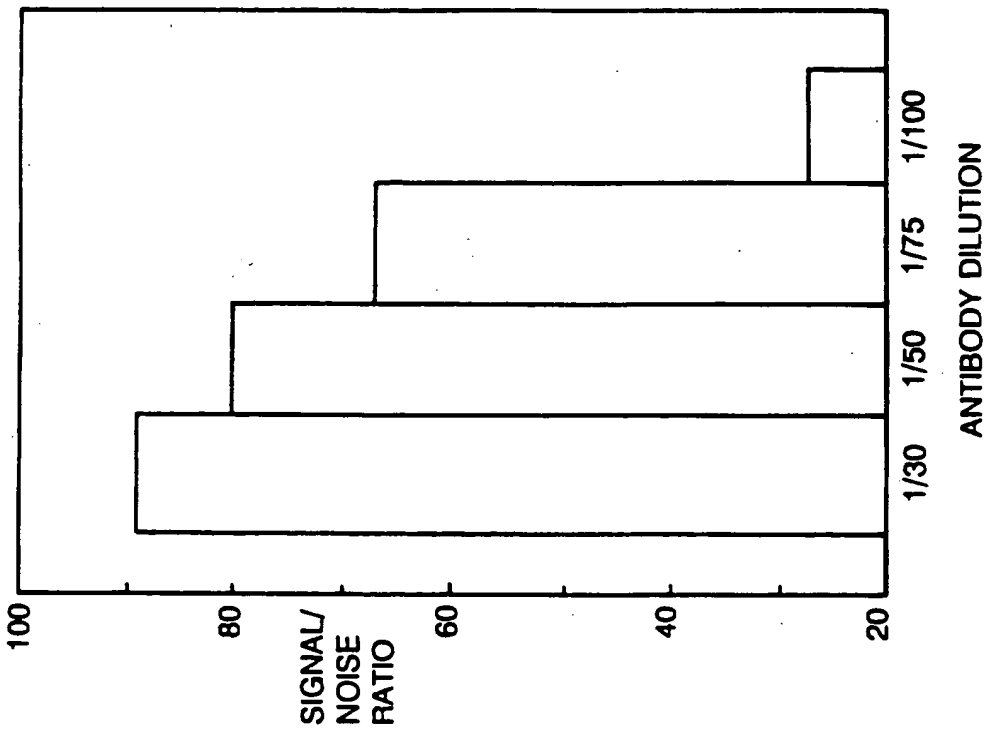

FIG. 3B

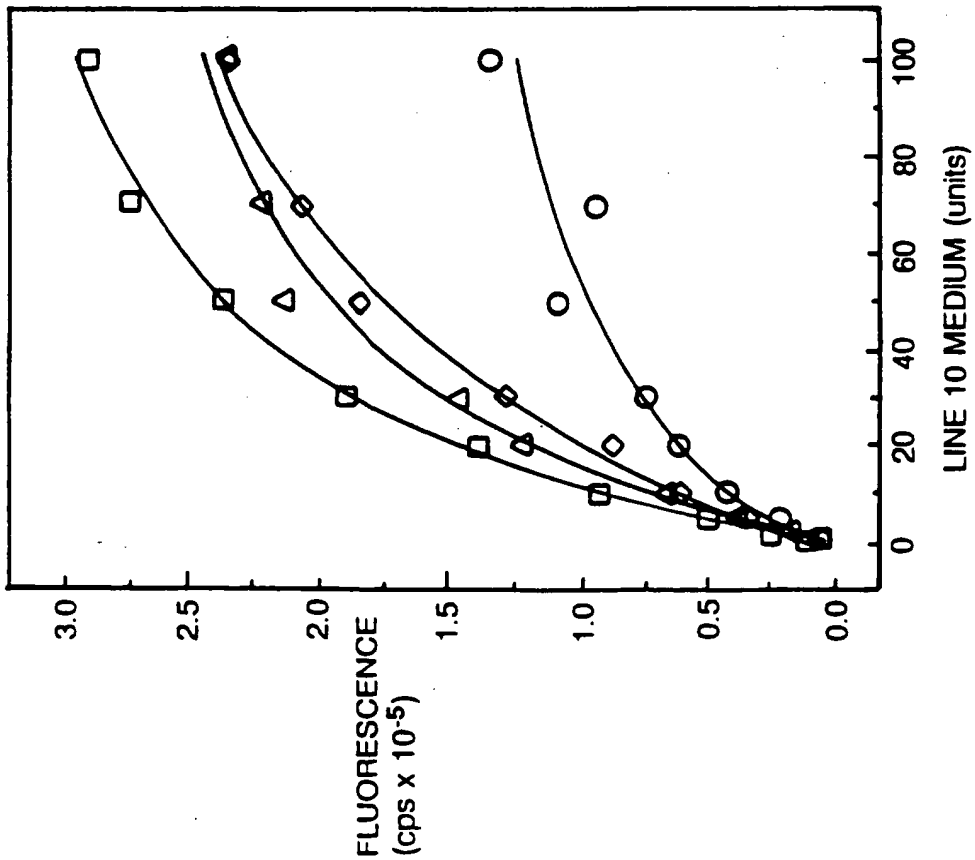

FIG. 3A

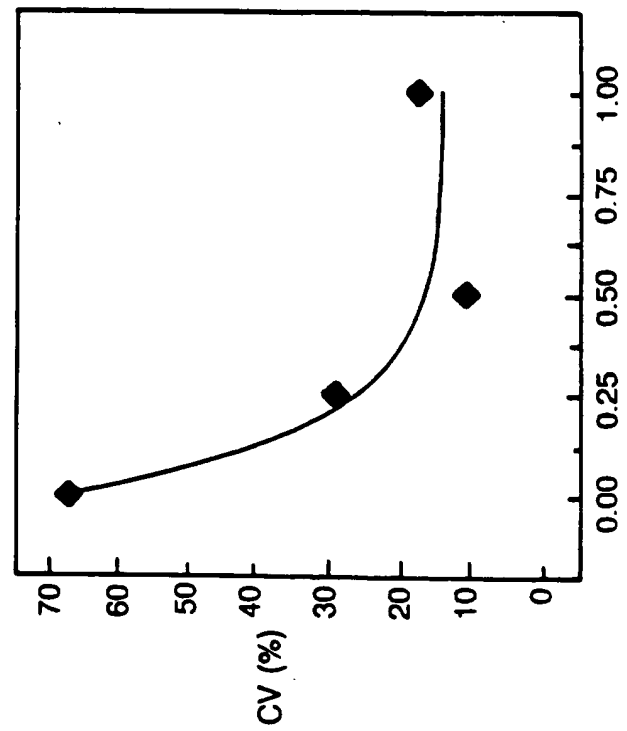
70
60
50
40
30
20
10
0
CV (%)
0.00
0.25
0.50
0.75
1.00
LINE 10 MEDIUM (units)

FIG. 4B

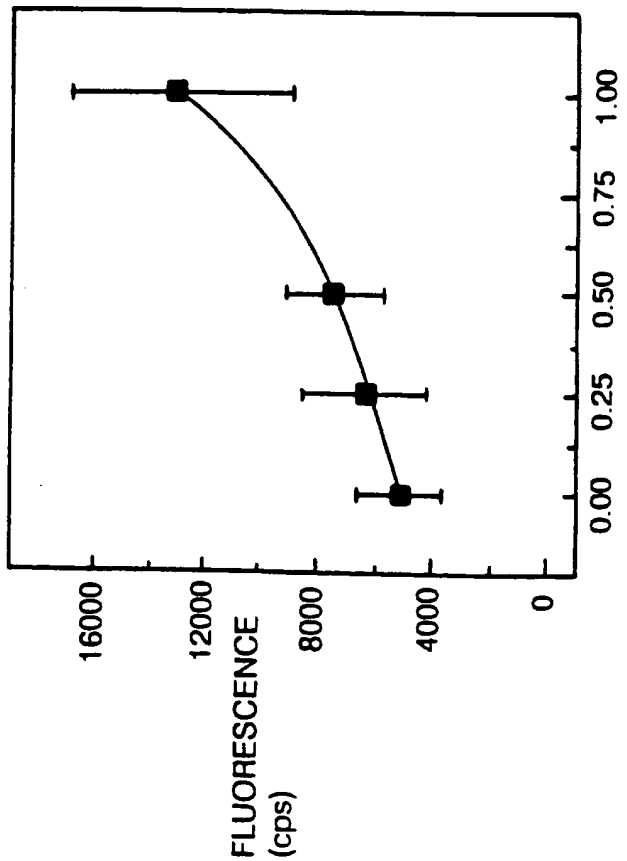
16000
12000
8000
4000
0
FLUORESCENCE (cps)
0.00
0.25
0.50
0.75
1.00
LINE 10 MEDIUM (units)

FIG. 4A

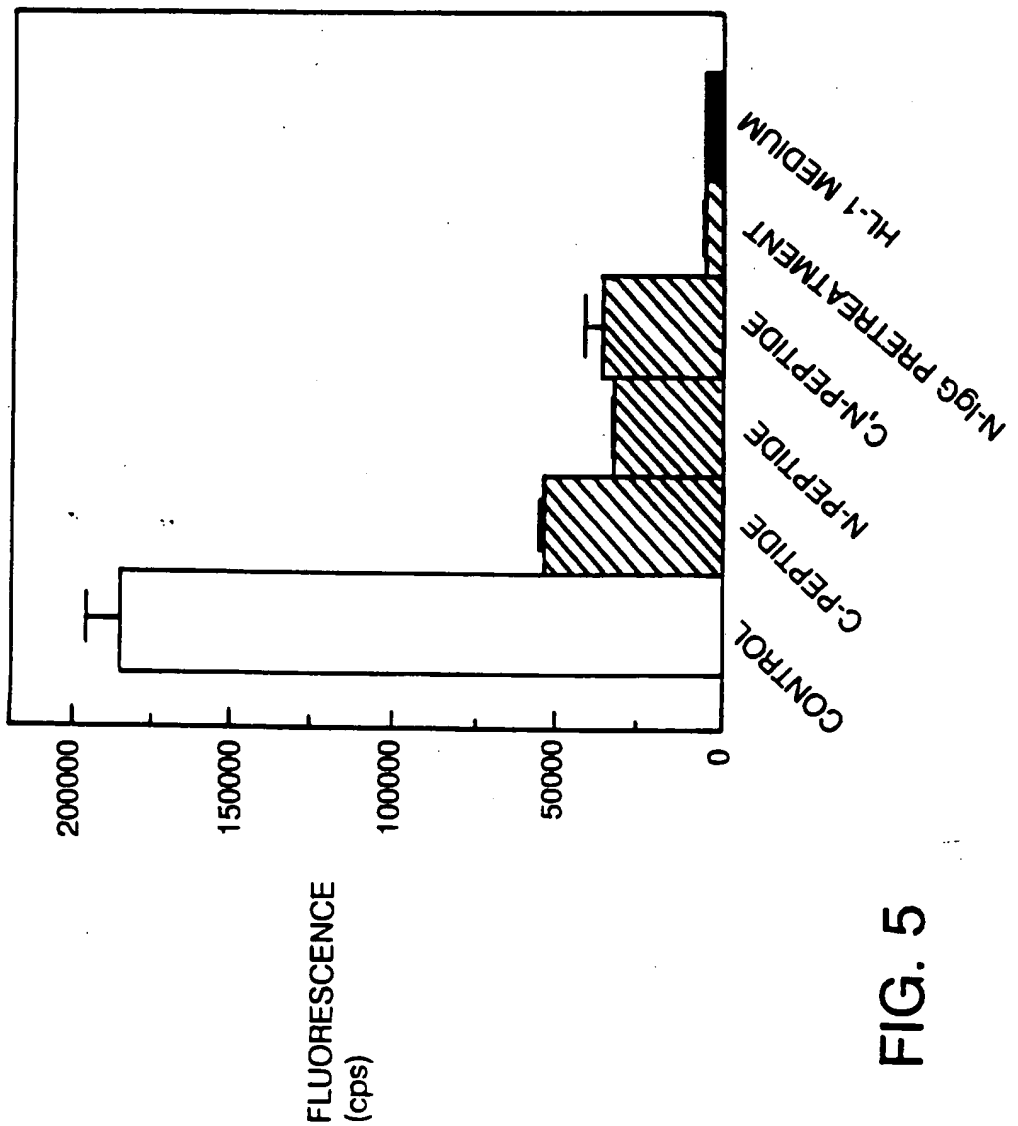

FIG. 5

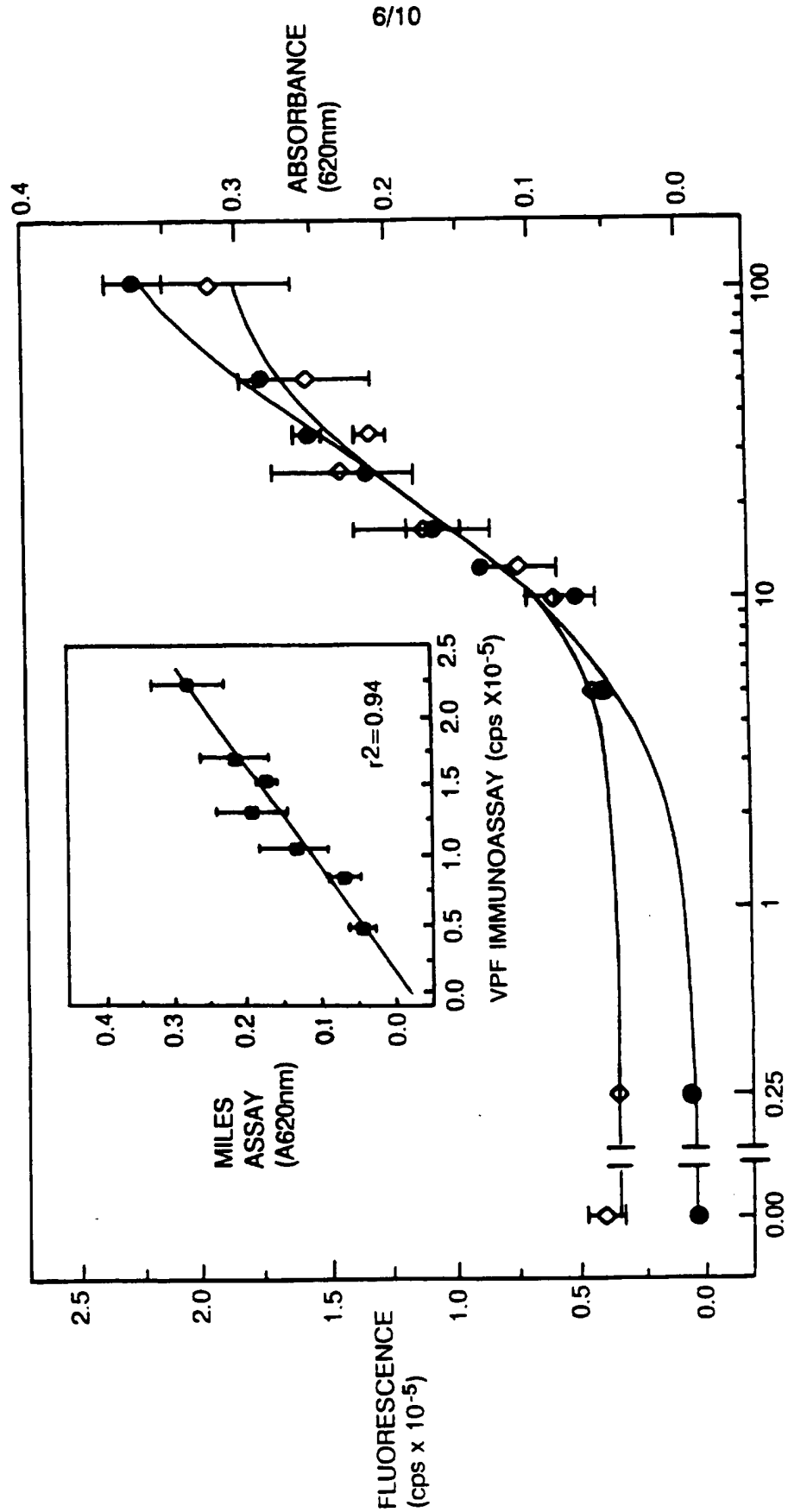
ABSORBANCE (620nm)
0.4
0.3
0.2
0.1
0.0
FLUORESCENCE (cps x 10-5)
2.5
2.0
1.5
1.0
0.5
0.0
[VPF] (units)
0.00
0.25
1
10
100
MILES ASSAY (A620nm)
VPF IMMUNOASSAY (cps X10-5)
r2=0.94

FIG. 6

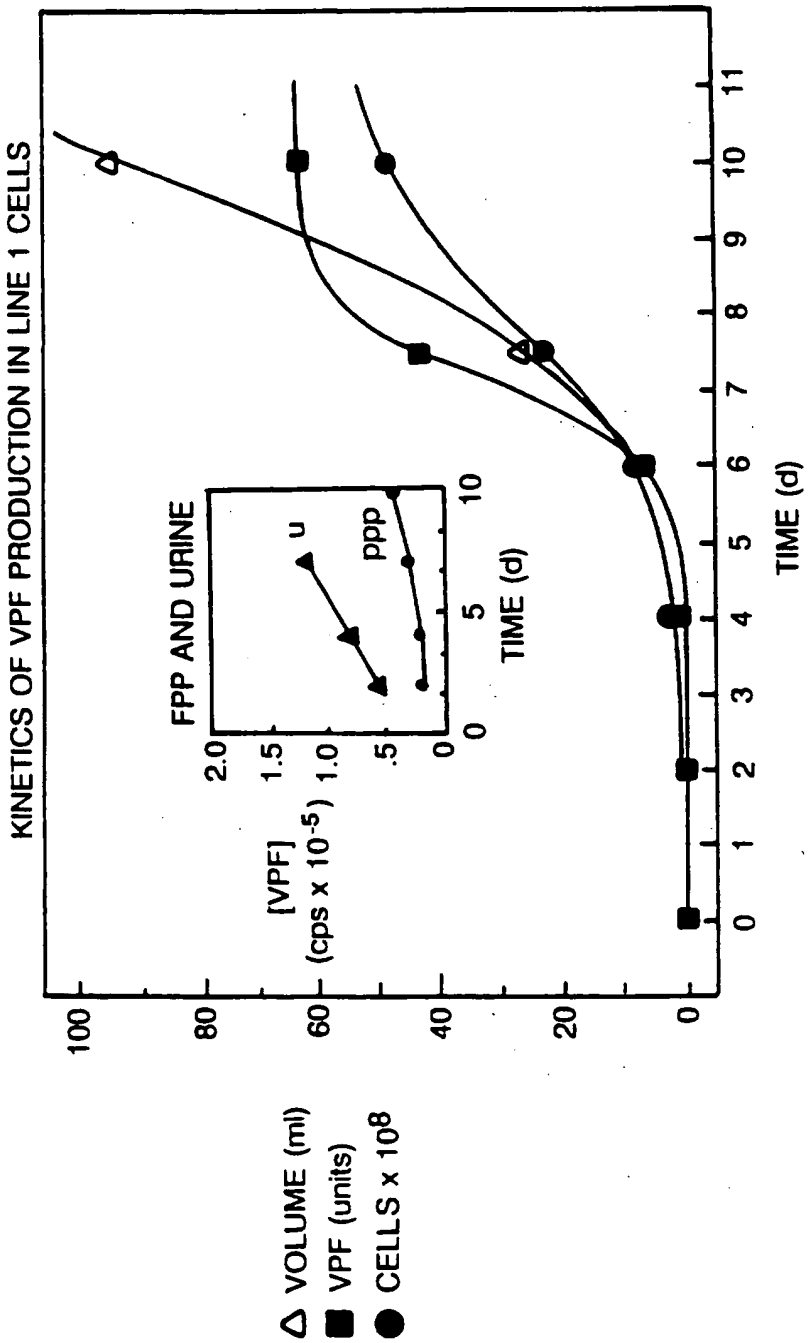
KINETICS OF VPF PRODUCTION IN LINE 1 CELLS
FPP AND URINE
[VPF] (cps x 10^{-5})
TIME (d)
u
ppp
Δ VOLUME (ml)
■ VPF (units)
● CELLS x 10^8
TIME (d)

FIG. 7

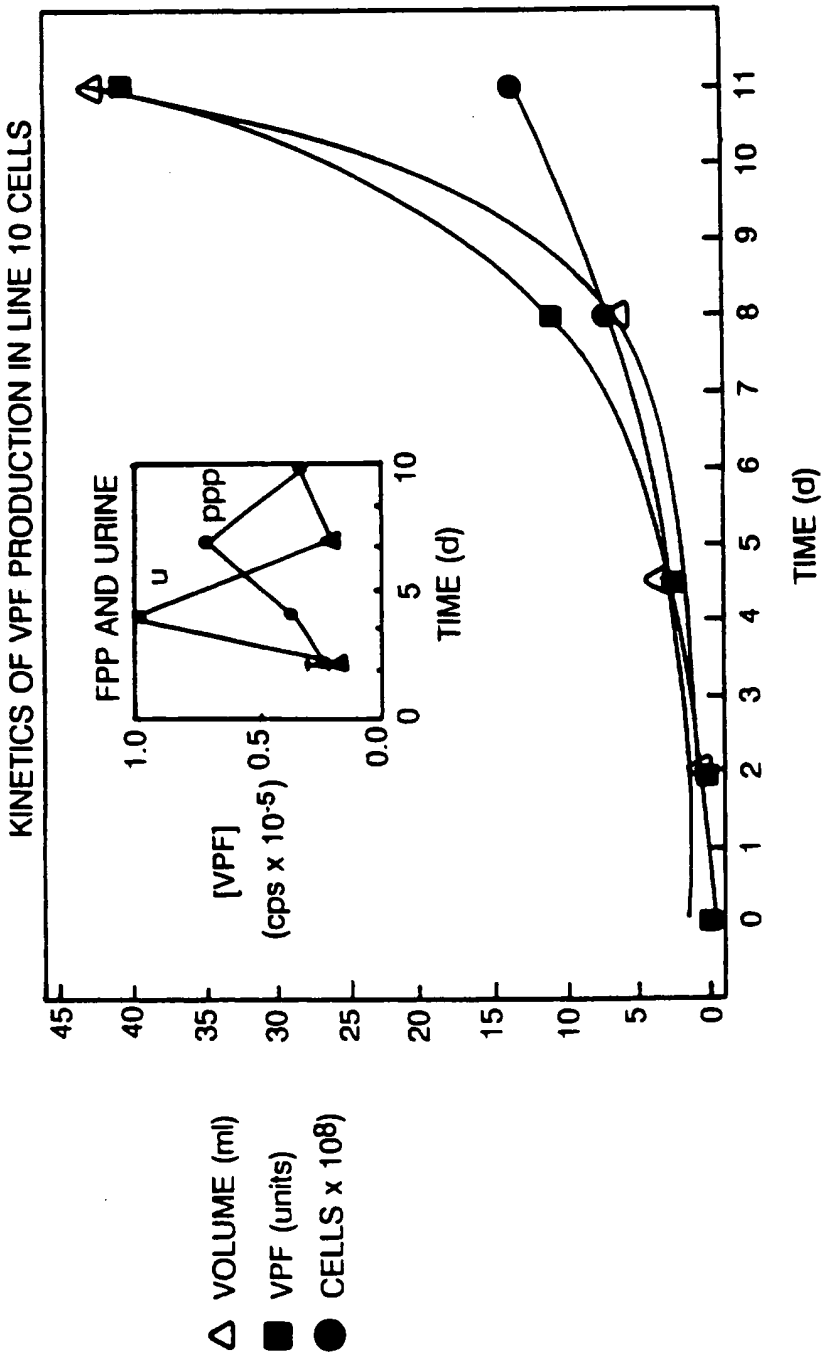
KINETICS OF VPF PRODUCTION IN LINE 10 CELLS
FPP AND URINE
u
ppp
[VPF]
(cps x 10-5)
1.0
0.5
0.0
0
5
10
TIME (d)
45
40
35
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11
TIME (d)
VOLUME (ml)
VPF (units)
CELLS x 10^8

FIG. 8

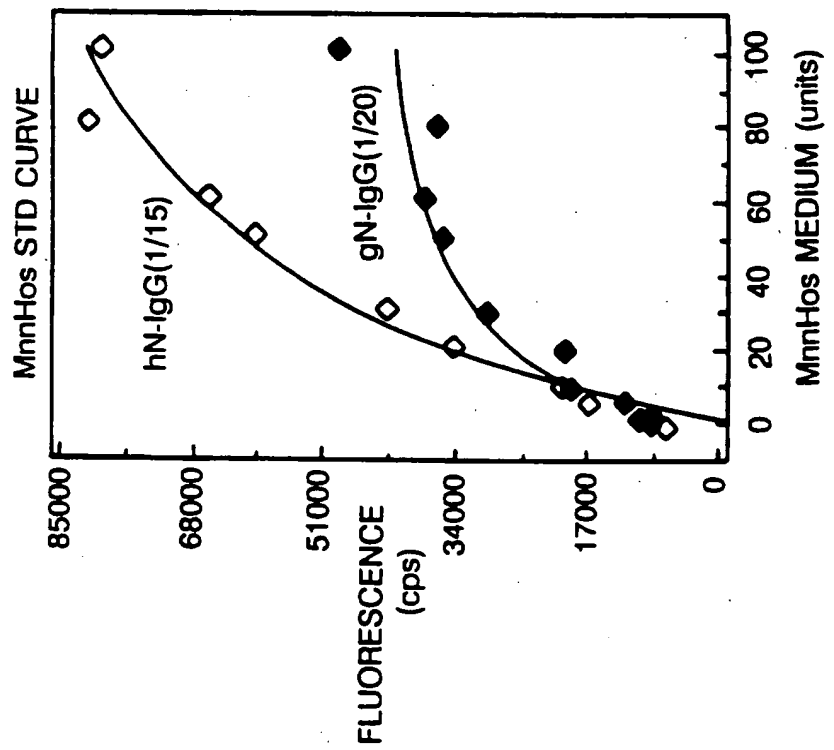

FIG. 9B

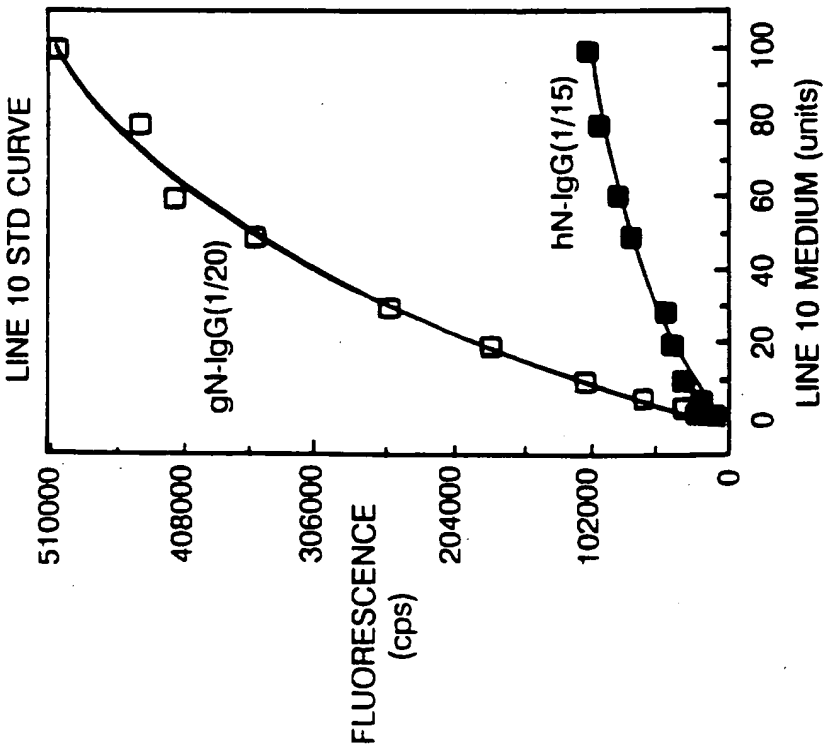

FIG. 9A

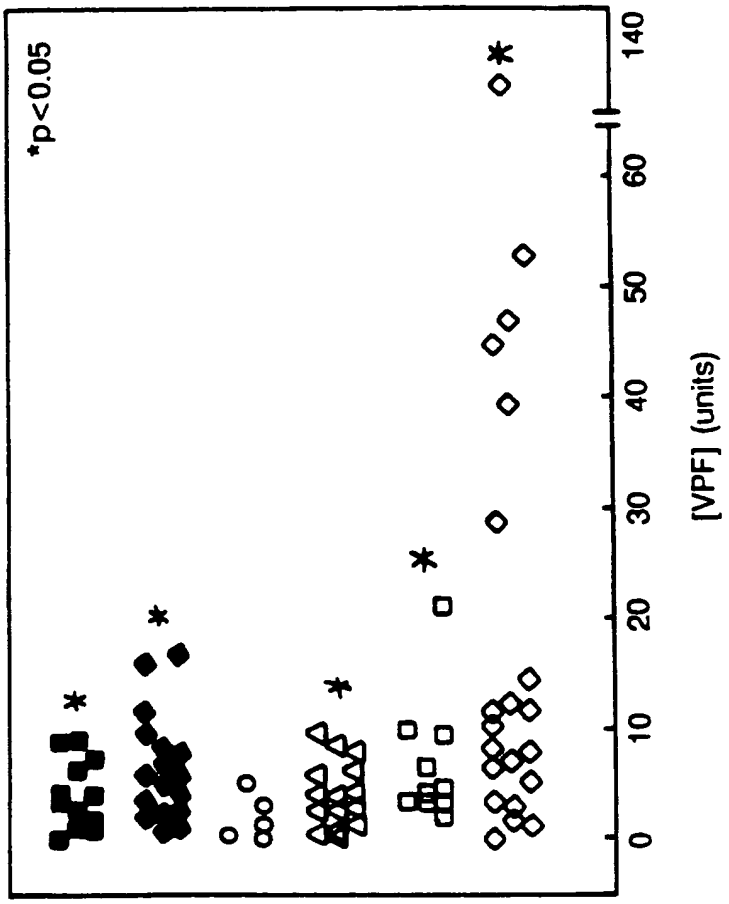

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09068

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/543, 33/50, 33/53
US CL :436/518, 64, 86
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/63; 530/387.1, 388.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, BIOSIS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, Volume 219, issued 25 February 1983 (WASHINGTON, DC, USA), D. R. SENGER ET AL, "Tumor Cells Secrete a Vascular Permeability Factor That Promotes Accumulation of Ascites Fluid", pages 983-985, especially page 985, first column, lines 5-10.	1-8
Y	CANCER RESEARCH, Volume 46, issued November 1986, (USA), D. R. SENGER ET AL., "A Highly Conserved Vascular Permeability Factor Secreted by a Variety of Human and Rodent Tumor Cell Lines", pages 5629-5632, especially page 5629, Abstract and first paragraph of the Introduction, and page 5632, last paragraph.	1-8
Y	US, A, 4,376,110 (DAVID ET AL.) 08 MARCH 1983, especially column 1, line 35 to column 2, line 7.	2, 4-8
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 264, No.33, issued 25 November 1989, (USA), D. T. CONNOLLY ET AL., "Human Vascular Permeability Factor. Isolation from U937 Cells", pages 20017-20024, especially 20019, first column, "Antibodies and Immunoassays" section.	2, 4-8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 23 November 1992	Date of mailing of the international search report 02 DEC 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer SHERYL K. REILLY Telephone No. (703) 308-3905

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ACTA CYTOLOGICA, Volume 32, issued September-October 1988, W. W. JOHNSTON, "Fine Needle Aspiration Biopsy Versus Sputum and Bronchial Material in the Diagnosis of Lung Cancer. A Comparative Study of 168 Patients", pages 641-646, especially 645, bridging paragraph between columns 1 and 2.	3
Y	SCANDANAVIAN JOURNAL OF CLINICAL LABORATORY INVESTIGATION, Volume 48, issued 1988, I. HEMMILA, "Lanthanides as probes for time-resolved fluorometric immunoassays", pages 389-399, especially page 392.	4
Y	SCIENCE, Volume 246, issued 08 December 1989, (WASHINGTON, DC, USA), P. J. KECK ET AL., "Vascular Permeability Factor, an Endothelial Cell Mitogen Related to PDGF", pages 1309-1312, especially page 1310, Figure 1.	5-8